Med1 plays a critical role in the development of tamoxifen resistance

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Understanding the molecular pathways that contribute to the development of tamoxifen resistance is a critical research priority as acquired tamoxifen resistance is the principal cause of poor prognosis and death of patients with originally good prognosis hormone-responsive breast tumors. In this report, we provide evidence that Med1, an important subunit of mediator coactivator complex, is spontaneously upregulated during acquired tamoxifen-resistance development potentiating agonist activities of tamoxifen. Phosphorylated Med1 and estrogen receptor (ER) are abundant in tamoxifen-resistant breast cancer cells due to persistent activation of extracellular signal-regulated kinases. Mechanistically, phosphorylated Med1 exhibits nuclear accumulation, increased interaction with ER and higher tamoxifen-induced recruitment to ER-responsive promoters, which is abrogated by inhibition of Med1 phosphorylation. Stable knockdown of Med1 in tamoxifen-resistant cells not only reverses tamoxifen resistance in vitro but also in vivo. Finally, higher expression levels of Med1 in the tumor significantly correlated with tamoxifen resistance in ER-positive breast cancer patients on adjuvant tamoxifen monotherapy. In silico analysis of breast cancer, utilizing published profiling studies showed that Med1 is overexpressed in aggressive subsets. These findings provide what we believe is the first evidence for a critical role for Med1 in tamoxifen resistance and identify this coactivator protein as an essential effector of the tamoxifen-induced breast cancer growth.

Introduction

Estrogen is a key regulator for normal growth and differentiation of mammary glands as well as the malignant progression of breast cancer (1). Consequently, interruption of estrogen signaling by using endocrine therapies has been an effective therapeutic strategy. Tamoxifen is the most prolific therapeutic drug for the treatment of estrogen receptor (ER)-positive breast cancer, showing effective tumor growth inhibition, reduced mortality and prevention of disease recurrence (2). Despite excellent efficacy, the use of tamoxifen is limited by the development of acquired resistance as a majority of initially responsive tumors later develop resistance to tamoxifen (3). Several molecular mechanisms have been proposed for the development of acquired resistance, including expression of variant or mutant ER, intimate cross talk between the ER and HER2/growth factor signaling pathway, altered cytoplasmic and genomic signaling of ER, alternative proliferative and survival stimuli (3,4). Recent studies have revealed that distinct coregulatory complexes modulate transcriptional activity of ER. Ligand-bound ER recruits multiple coactivator/corepressor complexes, which serve as a bridge between ER and the general transcription machinery or associate with histone acetyltransferases or histone deacetylases (5). While the role of coregulatory complexes for ER is very well established in mediating genomic signaling of ER in response to different ligands during normal breast development and tumor progression, the importance of coregulators in development of tamoxifen resistance is still under investigation.

Coactivators that directly interact with liganded ER and facilitate transactivation include members of the p160/steroid receptor coactivator family (6). These proteins are thought to function in part by associating with potent histone acetyltransferases resulting in acetylation of nucleosomes and transactivation of ER-responsive genes (6). Another important, conserved multisubunit coactivator complex is a mediator complex that binds to liganded ER and plays a key regulatory role (7). Mediator complex positively influences ligand-dependent transactivation by interacting with basal transcription machinery, possibly by directly facilitating the recruitment of RNA polymerase II (8). Mediator complex is comprised of ~30 subunits arranged in four subcomplexes known as the head, middle, tail and cdk8-containing modules (9,10). Mediator complex is directed to liganded ER by its anchor subunit, Med1, via two signature LXXLL motifs that serve as nuclear receptor-binding surfaces (11). Adding to its functional importance in regulating cell growth and development, Med1 also serves as a direct binding target for other regulatory transcription factors such as the GATA family of proteins, GABPα, BRCA-1 and p53 (12–17).

In this study, we present evidence that Med1 overexpression renders ER-positive breast cancer cells resistant to tamoxifen. Elevated expression of Med1, along with a significant increase in Med1 and ER phosphorylation due to persistent activation of extracellular signal-regulated kinase (ERK) in tamoxifen-resistant cells, leads to increased recruitment of ER-Med1 complex to ER-responsive genes upon tamoxifen treatment. Using isogenic Med1-knockdown tamoxifen-resistant cell line pair, we show that Med1 is required for tamoxifen-induced growth of tamoxifen-resistant cells in vitro and in vivo. Analysis of clinical samples showed that high Med1 expression is significantly linked to tamoxifen resistance in breast cancer. Also, in silico analyses of multiple independent microarray expression datasets revealed Med1 overexpression in subsets of breast tumors correlated with increased aggressiveness, higher clinical risk and mortality. Therefore, Med1 could serve as a critical regulator altering ER-mediated transcription rendering cells nonresponsive to tamoxifen, thus mediating the development of tamoxifen-resistant phenotypes.

Materials and methods

Reagents, hormones and antibodies

17β-estradiol (E2), 4-hydroxytamoxifen (OHT), epidermal growth factor (EGF) and U0126 were from Sigma (St Louis, MO). Anti-Med1, anti-phospho-Med1 (Abcam), anti-ER (Santa Cruz), anti-phospho-ER (Serine167), anti-phospho-ER (Serine118), anti-phospho-ER and anti-ERK (Cell Signalling) antibodies were used.

Cell culture

The human breast cancer cell line, MCF7, was obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gemini Bio-products, Woodland, CA) (18). Cell line authentication was done by analysis of known
med1 mediates tamoxifen resistance in breast cancer

Chromatin immunoprecipitation lysates were incubated with specific antibodies for Med1 followed by incubation with protein A/G agarose beads. The precipitated protein-bead complexes were subjected to immunoblotting.

Immunofluorescence and confocal imaging

To determine the role of ERK phosphorylation in Med1 localization, cells were treated for 2 h with 100 ng/ml EGF (Sigma–Aldrich) or 30 μM U1026 (Sigma–Aldrich) followed by tamoxifen treatment for 2 h. Cells (5 × 10⁶ cells per well) were plated in four-well chamber slides (Nunc, Rochester, NY), treated with EGF or U1026 and subjected to immunofluorescence analysis as described earlier (22). Fixed and immunofluorescently stained cells were imaged using a Zeiss LSM510 Meta (Zeiss) laser scanning confocal system configured to a Zeiss Axioplan 2 upright microscope with a 63XO (NA 1.4) plan-achromat objective. Cells were observed at five randomly selected fields in single focal plane for Med1 localization. All experiments were performed multiple times using independent biological replicates.

med1 stable knockdown using lentiviral short hairpin RNA

Five premade lentiviral Med1 short hairpin RNA (shRNA) constructs and a negative control construct created in the same vector system (pLKO.1) were purchased from Open Biosystems (Huntville, AL). Paired Med1 stable knockdown cells were generated following our previously established protocol (22). Lentiviral helper plasmids (pCMV-dR8.2 dvpr and pCMV-NSV-G) were obtained from Addgene (Cambridge, MA). Transient lentivirus stocks were prepared following the manufacturer’s protocol. One day before transfection, 1.5 × 10⁵ MCF7 control cells were plated in 100 mm dishes. Cells were cotransfected with shRNA constructs (3 μg) together with 3 μg pCMV-dR8.2 dvpr and 0.3 μg pCMV-NSV-G helper constructs. Two days later, viral stocks were harvested from the culture medium and filtered to remove nonadherent 293T cells. To select for the MCF7-P and MCF7-OHT cells that were stably expressing shRNA constructs, cells were plated at subconfluent densities and infected with a cocktail of 1 ml of virus-containing medium, 3 ml of regular medium and 8 μg/ml polybrene. Selection with 0.5–2 μg/ml of puromycin was started 48 h after lentivirus infection. After 4 weeks of selection for MCF7-P and MCF7-OHT cells, monolayers of stably infected pooled clones were harvested for use and cryopreserved.

Athylic nude mice model for tamoxifen-resistant growth

Four- to six-week-old female athylic nude mice (Harlan Laboratories Inc., Indianapolis, IN) were given a subcutaneous injection of 5 × 10⁶ cells (50% phosphate-buffered saline/50% Matrigel in a total volume of 50 μl). When tumors reached the size of 50 mm³, the animals were randomly allocated (n = 10 per group) to various groups. Vehicle and tamoxifen treatment (500 μg of tamoxifen citrate in peanut oil by subcutaneous injection daily Monday to Friday, for 6 weeks) were given for respective groups. Estrogen treatment was provided by the ethical committees at Lund and Linköping Universities. All patients received surgery followed by radiotherapy if indicated and only patients with no recurrent breast cancer event was 13.9 months. Several tamoxifen-resistant (MCF7-OHT) clones of MCF7-P cells formed multiple times using independent biological replicates.

immunohistochemistry of clinical specimens

From 1986 until 1991, 564 premenopausal patients under 50 years with stage II (pT2 N0 M0, pT1 N1 M0 and pT2, N1 M0) invasive breast cancer were enrolled in a trial (SBII.2a) and randomized to either 2 years of adjuvant tamoxifen (n = 276) or no systemic adjuvant treatment (control) (n = 288). All patients received surgery followed by radiotherapy if indicated and only 2% (n = 9) of the patients received adjuvant chemotherapy. The median follow-up time for patients with no recurrent breast cancer event was 13.9 years. Details of this trial have been described (30). This study was approved by the ethical committees at Lund and Linköping Universities.

Analysis/scoring of Med1. Med1 expression was assessed immunohistochemically as absent = 0, very low = 1, low = 2, intermediate = 3, intermediate high = 4, high = 5 in combination with fraction of positive nuclei/cytoplasm where 0 = 0–1%, 1 = 1–10%, 2 = 11–25%, 3 = 26–50% and 4 = 51–100%. Med1 IHC analysis was scored using a combined analysis of nuclear/cytoplasmic intensity and fraction positive cells into 0–5 where 4–5 represented >50% cells showing staining of moderate or strong intensity. Since overexpression of Med1 in breast cancer was the scope of the examinations, we focused on the two highest subgroups of nuclear staining versus low expression. Cytoplasmic Med1 expression was scored into mo/low expression or high. All immunohistochemical analyses were performed by a pathologist (L.I.).
Table 1, available at Carcinogenesis (Online) were analyzed for Med1 expression levels.

Statistical analysis
All experiments were independently performed three times in triplicates. Statistical analysis was done using Microsoft Excel software. Significant differences were analyzed using student’s t-test and two-tailed distribution. Data were considered to be statistically significant if P < 0.05. Data are expressed as means ± SE between triplicate experiments. The distribution of clinicopathological characteristics by Med1 expression was evaluated using Fisher’s exact tests, Spearman’s ρ or Pearson chi-square tests. Continuous variables (age) were analyzed using Mann–Whitney U tests. Survival analyses were generated using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios (HRs) were calculated using Cox proportional hazards regression. Statistical analyses were performed using SPSS software (version 15.0.1; Chicago, IL).

Results
ER function is modified in the acquisition of tamoxifen resistance
To understand how tamoxifen resistance develops in initially responsive ER-positive breast cancer cells, we developed isogenic cell line pairs (Supplementary Figure 1A, available at Carcinogenesis (Online)) exhibiting acquired tamoxifen resistance. In vivo, MCF7-OHT tumors grow rapidly in the presence of tamoxifen, whereas MCF7-P tumors get inhibited by tamoxifen treatment (Figure 1A). MCF7-P cells showed increased proliferation, clonogenicity and 3-D colony formation in response to estrogen treatment. MCF7-P cells form smaller colonies in the absence of estrogen while estrogen treatment enhances the number as well as the size of colonies. Tamoxifen treatment efficiently inhibited growth and clonogenicity of MCF7-P cells, whereas MCF7-OHT cells continued to grow at an increased rate (Figure 1B and Supplementary Figure 1B and C, available at Carcinogenesis Online). The resistant clones typically form similar number of colonies (containing >50 cells) as parent cells upon estrogen stimulation. However, the colonies of parent cells continue to grow in response to estrogen while resistant clones form smaller colonies displaying reduced response to estrogen stimulation. Change in ER level may modulate estrogen and tamoxifen response. However, immunoblot analysis showed similar levels of ER protein levels in both MCF7-P and MCF7-OHT cells (Figure 1C).

Estrogen signaling regulates various biological processes such as cell cycle progression, proliferation, signaling pathways and apoptosis in normal as well as malignant growth via regulation of expression of various important cellular oncogenes and tumor suppressor genes. Overexpression/over-representation of E2-responsive genes has been associated with enhanced growth and aggressiveness of breast cancer (31, 32). Tamoxifen-mediated inhibition of these E2-responsive genes in MCF7 cells collectively leads to breast cancer growth inhibition. In contrast, tamoxifen activates the expression of these E2-responsive genes in tamoxifen-resistant cells altering the molecular network to enhance breast tumor growth. Steroid receptors, such as ER, regulate gene transcription either by binding directly to the promoter of target genes or by binding indirectly through other transcription factors (1, 33). Genes regulated through direct ER binding, such as EBAG9 (ER-binding fragment-associated antigen 9), cathepsin D and PS2, typically harbor a classic hormone-responsive element (34, 35). In contrast, genes, such as c-Myc and IGF1, are regulated by indirect binding of ER to non-classic HREs (36, 37). EBAG9, cathepsin D and PS2 were chosen to represent hormone-responsive element-containing target genes while c-Myc and IGF1 represented non-hormone-responsive element-containing target genes.

Examination of tamoxifen-mediated changes in expression pattern of endogenous ER-responsive genes (cathepsin D, c-myc, EBAG9, PS2 and IGF1) revealed inhibited expression upon tamoxifen treatment in MCF7-P cells, whereas MCF7-OHT cells showed increased expression in response to tamoxifen (Figure 1D and E). Furthermore, estrogen increased while tamoxifen inhibited ER-mediated transactivation in MCF7-P cells. MCF7-OHT cells showed significantly elevated levels of ER transactivation function in response to tamoxifen while showing an increased luciferase activity in response to estrogen albeit at a lower level in comparison with tamoxifen (Figure 1F). Collectively, these studies showed that the acquisition of tamoxifen resistance is associated with differential sensitivity to estrogen and tamoxifen, retention of ER protein while modifying its transactivation function in order to differentially alter expression of ER-responsive genes in response to tamoxifen.

Alteration in expression and phosphorylation of Med1 and ER due to persistent activation of ERK modulates their functional interaction in acquired tamoxifen resistance
We discovered that Med1 was overexpressed in tamoxifen-resistant MCF7-OHT cells in comparison with parental tamoxifen-sensitive MCF7-P cells (Figure 2A). Med1 facilitates functional interactions between regulatory transcription factors and the general polymerase II initiation apparatus during ER-responsive gene regulation (9). Coregulatory functions of human Med1 may get modulated via altered phosphorylation. Interestingly, we observed persistent activation of ERK in MCF7-OHT cells indicating a possible role of ERK in tamoxifen resistance (Figure 2A). We hypothesized that Med1 phosphorylation may get altered by MAPK-ERK and acting as a regulatory mechanism may influence its association with the core mediator complex along with its coactivation functions. Increased accumulation of phosphorylated Med1 was observed in MCF7-OHT cells in comparison with MCF7-P cells (Figure 2A). EGF treatment is known to activate ERK (19). Stimulation with EGF (Figure 2B) or cotransfection with the MKK1 (NA4) or ERK2 (L73P/S151D) (38) expression constructs (data not shown) markedly enhanced Med1 phosphorylation, whereas addition of U0126, an inhibitor for ERK, inhibited Med1 phosphorylation (Figure 2B). Phosphorylation modifications of ER have been implicated in important regulatory function of ER. ER is phosphorylated at Ser-167 and Ser-118 in response to active ERK signaling (39). MCF7-OHT cells showed increased phosphorylation of ER at Ser-167 and Ser-118 sites (Figure 2C). ER phosphorylation at both Ser-167 and Ser-118 was further enhanced in response to EGF stimulation, whereas U0126 treatment led to inhibition of ER phosphorylation (Figure 2D).

Activation of ERK pathway brings additional complexity because the ability of coactivators to interact with nuclear hormone receptors and influence transcription can be affected by their altered phosphorylation. In immunoprecipitation assay, MCF7-P cells exhibited Med1:ER interaction upon E2 treatment, whereas no interaction was observed in the presence of tamoxifen. In contrast, Med1 and ER showed a substantial interaction in a ligand-independent manner which was increased with E2 and tamoxifen treatment in MCF7-OHT cells (Figure 3A). In an effort to better understand the molecular events involved in acquired tamoxifen resistance, we used ChIP analyses to examine the recruitment of Med1 to specific ER-responsive gene promoters in response to tamoxifen treatment. ER got recruited to ER-responsive gene promoters in response to tamoxifen in MCF7-P and MCF7-OHT cells (Figure 3B, C). Coactivator Med1 did not bind to EREs in the presence of tamoxifen in MCF7-P cells. Intriguingly, tamoxifen treatment induced recruitment of Med1 to ER-responsive gene promoters in tamoxifen-resistant MCF7-OHT cells (Figure 3B, C). Med1 phosphorylation significantly increased in tamoxifen-resistant cells (Figure 3B, C). Tamoxifen inhibited the expression of ER-responsive genes, cathepsin D, c-myc, EBAG9, PS2 and IGF1 in MCF7-P cells, whereas MCF7-OHT cells showed increased expression upon tamoxifen treatment (Figure 5D and Supplementary Figure 3, available at Carcinogenesis Online).

Next, we determined the impact of phosphorylation of Med1 on its nuclear translocation and tamoxifen-induced recruitment to ER-responsive promoters in tamoxifen-resistant cells. Med1 is majorly localized in nucleus in MCF7-OHT cells (Figure 4A and B). Treatment with U0126 reduces the total amount of Med1 indicating that the ERK pathway regulates Med1 protein expression (Figure 4A). Nuclear localization of Med1 is greatly enhanced in response to EGF stimulation (Figure 4A and B), which has been shown to increase its phosphorylation (Figure 2). ChIP analyses revealed that tamoxifen induced recruitment of ER and Med1 to ER-responsive genes which was abrogated by U0126 treatment (Figure 4C and D). Collectively,
our findings suggest that persistent activation of ERK in tamoxifen-resistant cells leads to elevated phosphorylation levels of ER and Med1 that may act as a regulatory mechanism to promote Med1 association with ER in response to tamoxifen. Further mechanistic studies directly examining the role of phosphorylation in MED1-ER interaction may show that altered phosphorylation of Med1 and ER may facilitate a novel feed-forward action of tamoxifen during acquired tamoxifen resistance. These evidence support the notion that tamoxifen induced increased Med1-ER interaction and increased Med1 recruitment at ER-responsive gene promoters in tamoxifen-resistant but not in tamoxifen-sensitive cells may be due to altered phosphorylation of Med1 and ER in tamoxifen-resistant cells.

**Targeted downregulation of Med1 in tamoxifen-resistant cells reverses acquired tamoxifen resistance**

To determine the importance of Med1 in tamoxifen resistance, endogenous Med1 was transiently knocked down in MCF7-P and MCF7-OHT cells using psilencer-Med1 (Supplementary Figure 2A is available at Carcinogenesis Online). In MCF7-OHT cells transfected with psilencer-control, tamoxifen treatment induced significant increases in tumor growth and clonogenicity and augmented ER-responsive gene expression and ER-mediated transactivation in tamoxifen-resistant cells. (A) Tumor growth curves of MCF7-P and MCF7-OHT cells implanted subcutaneous in athymic mice in the presence of estrogen and tamoxifen treatment. (B) Anchorage-dependent growth of MCF7-P and MCF7-OHT cells in the presence of various doses of 17β-estradiol (E2) or 4-OHT. Colonies containing ≥50 normal-appearing cells were counted. *, P < 0.001, compared with untreated controls (MCF7-P and MCF7-OHT); #, P < 0.001, compared with untreated controls (MCF7-P and MCF7-OHT). (C) Immunoblot analysis of ER protein in MCF7-P and MCF7-OHT cells. (D) Semi-quantitative reverse transcription–PCR analysis of messenger RNA (mRNA) expression levels of cathepsin-D and c-Myc mRNA in MCF7-P and MCF7-OHT cells. (E) Real-time quantitative PCR analysis of EBAG9, pS2 and IGF1 expression in MCF7-P and MCF7-OHT cells treated with 1 μM of 4-OHT for 2 h. *, P < 0.005, compared with untreated controls (MCF7-P); #, P < 0.001, compared with untreated controls (MCF7-OHT). (F) Luciferase activity of pERE-Luc in MCF7-P and MCF7-OHT cells treated with either ethanol vehicle alone or 100 nM E2 or 1 μM OHT for 24 h. *, P < 0.001, compared with untreated controls (MCF7-P and MCF7-OHT); **, P < 0.02, compared with untreated controls (MCF7-P); #, P < 0.001, compared with untreated controls (MCF7-OHT).
growth. Interestingly, Med1 inhibition in MCF7-OHT cells significantly reduced tamoxifen-induced growth (Supplementary Figure 2B is available at Carcinogenesis Online) indicating the importance of Med1 in tamoxifen resistance while not affecting growth in the absence of tamoxifen. Next, we used Med1 shRNA and pLKO.1 (vector control) lentivirus and puromycin to select for stable pools of MCF7-P and MCF7-OHT cells with stable Med1 depletion. Med1-depleted (Med1 shRNA) MCF7-P and MCF7-OHT cells showed efficient knockdown of Med1 in comparison with vector control (pLKO.1) cells (Figure 5A). Examination of clonogenic potential and 3-D soft agar colony formation of MCF7-P-pLKO.1 and MCF7-P-pMed1 shRNA cells in the presence of tamoxifen revealed that Med1 inhibition did not affect tamoxifen-mediated inhibition of MCF7-P cells. While knockdown of Med1 in MCF7-OHT (MCF7-OHT-pMed1 shRNA) cells significantly inhibited tamoxifen-induced clonogenicity and 3-D soft agar colony formation in comparison with vector control (MCF7-OHT-pLKO.1) cells (Figure 5B and C). We also examined the effect of Med1 depletion on ER-responsive gene expression. Expression of ER-responsive genes was inhibited in response to tamoxifen treatment in MCF7-P-pLKO.1 and MCF7-P-pMed1 shRNA cells while MCF7-OHT-pLKO.1 cells showed increased expression of cathepsin D, c-myc, EBAG9, p52 and IGF1 upon tamoxifen treatment. Inhibition of Med1 in tamoxifen-resistant cells (MCF7-OHT-pMed1 shRNA) reversed tamoxifen resistance leading to tamoxifen-mediated inhibition of expression of ER-responsive genes (Figure 5D and Supplementary Figure 3 is available at Carcinogenesis Online). Examination of four additional tamoxifen-resistant clones (Supplementary Figure 1 is available at Carcinogenesis Online) also showed higher Med1 expression along with increased ERK phosphorylation (data not shown). Silencing of Med1 in these tamoxifen-resistant clones inhibited tamoxifen-induced growth (data not shown). To strengthen and generalize these findings, we next investigated the impact of Med1 on tamoxifen resistance using two different established models of tamoxifen-resistant breast cancer cells (LCC2 and MCF7-5/23) (20,21). We found higher Med1 expression in tamoxifen-resistant LCC2 and MCF7-5/23 cells (Figure 6A and C) in comparison with their tamoxifen-sensitive counterparts. Also, stable Med1 depletion using Med1 shRNA lentivirus in LCC2 and MCF7-5/23 cells led to reversal of tamoxifen resistance further corroborating the importance of Med1 in tamoxifen resistance (Figure 6B and D).

We next examined the importance of Med1 in tamoxifen resistance in vivo. MCF7-OHT-pLKO.1 tumors showed marked increase in tumor growth in the presence of tamoxifen treatment as compared with untreated (Figure 7A and B). MCF7-OHT cells also showed an increased tumor growth in response to tamoxifen treatment (data not shown). Importantly, MCF7-OHT-pMed1 shRNA tumors exhibited significant inhibition of tumor growth in the presence of tamoxifen treatment showing that Med1 knockdown efficiently reversed tamoxifen resistance (Figure 7A and B). MCF7-OHT-pMed1 shRNA tumors always appeared less vascularized. The Ki-67 antigen is a useful marker of cell proliferation (40). Upregulation of proliferation marker, Ki67, was observed in tamoxifen-treated MCF7-OHT-pLKO.1 tumors in comparison with tamoxifen-treated MCF7-OHT-pMed1 shRNA tumors (Figure 7C). We further analyzed the alterations in ER-responsive gene expression in tumor samples and found overexpression of c-myc and cathepsin D in tamoxifen-treated MCF7-OHT-pLKO.1 tumors. Tamoxifen treatment did not induce expression of c-myc and cathepsin D in MCF7-OHT-pMed1 shRNA tumors (Figure 7D).

Fig. 2. Increased expression and phosphorylation of Med1 in tamoxifen-resistant cells due to persistent activation of ERK. (A) Immunoblot analysis of Med1, ERK, phospho-Med1 and phospho-ERK in MCF7-P and MCF7-OHT. (B) Immunoblot analysis of pMed1, pERK and ERK in MCF7-P and MCF7-OHT cells treated with EGF and U1026. (C) Immunoblot analysis of ER, p-ER (S-167) and p-ER (S-118) in MCF7-P and MCF7-OHT cells. (D) Immunoblot analysis of ER, p-ER (S-167) and p-ER (S-118) in MCF7-P and MCF7-OHT cells treated with EGF and of U1026.
Immunohistochemistry analysis of tumor samples showed increased expression of ER-responsive gene, progesterone receptor (PR) in tamoxifen-treated MCF7-OHT-pLKO.1 tumors in comparison with tamoxifen-treated MCF7-OHT-pMed1shRNA tumors (Figure 7E).

**Increased Med1 expression associates with higher tumor grade, increased clinical risk and recurrence after tamoxifen treatment**

Our observation that tamoxifen-resistant cells exhibit increased Med1 expression and targeted downregulation of Med1 in tamoxifen-resistant cells reverses acquired tamoxifen resistance *in vitro* and *in vivo* raised the question whether Med1 might play an important role in the tamoxifen resistance in human breast cancer. If this were the case, we reasoned that women with breast cancers expressing higher levels of Med1 might relapse after tamoxifen treatment. We examined Med1 protein levels of hormone receptor-positive breast cancers in a cohort of 564 patients treated with adjuvant tamoxifen (*n* = 276) or no systemic treatment (*n* = 288) (30). Figure 8A shows representative immunohistochemical staining of Med1 in breast tumors indicating variations in positivity among tumor cells. In these discovery sets of tissues, statistically significant association between higher expression of Med1 and poorer recurrence-free survival was observed (HR = 0.65, 95% confidence interval 0.36–0.90, *P* = 0.005). In the Med1 low subgroup, patients treated with tamoxifen had a significantly better recurrence-free survival compared with control patients in the same subgroup (HR = 0.45, 95% confidence interval 0.28–0.74, *P* = 0.001) (Figure 8B and Supplementary Figure 4, available at *Carcinogenesis* Online). These data suggested that elevated expression levels of total Med1 (both nuclear and cytoplasmic) associate with the development of tamoxifen resistance in breast cancer patients. Although our *in vitro* data suggest that Med1 has to act in the nucleus to mediate tamoxifen resistance, further studies using larger set of tissues are required to examine if high nuclear or cytoplasmic Med1 expression levels alone associate with tamoxifen resistance.

**Fig. 3.** Med1 associates with ER and tamoxifen increases recruitment of Med1 to ER-responsive gene promoters. (A) Immunoprecipitation of Med1 protein from MCF7-P and MCF7-OHT cells followed by immunoblot analysis of ER. (B) ChIP was performed using antibodies specific for ER and Med1 from MCF7-P and MCF7-OHT cells untreated and treated with 4-OHT (1 μM for 2 h). The purified DNA was analyzed by PCR using specific primers spanning the EREs of cathepsin D and c-myc gene promoters. (C) ChIP was performed as in (B). The purified DNA was analyzed by real-time quantitative PCR analysis using specific primers spanning the EREs of EBAG9, pS2 and IGF1 gene promoters. **P** < 0.001, compared with MCF-P treated with OHT.
resistance. In addition, we performed in silico analysis of breast cancer utilizing published profiling studies for Med1 expression levels. Analysis of hormone receptor-positive, early-stage invasive breast cancers from 60 women uniformly treated with adjuvant tamoxifen alone (41) showed a distinct subset of tumors with tamoxifen recurrence exhibited higher Med1 expression (Supplementary Figure 5A, available at Carcinogenesis Online). We also found significant association between higher Med1 expression and low survival; $P$ value $= 0.02$ and HR $= 1.54$. Patients with higher Med1 expression were at greater risk of mortality than patients with low Med1 expression (Supplementary Figure 5B, available at Carcinogenesis Online). Also, higher Med1 expression was associated with high tumor grade; $P$ value $= 0.029$ (Supplementary Figure 5C, available at Carcinogenesis Online).

Collectively, these studies showed that Med1 overexpression is indispensable for acquired tamoxifen resistance as ablation of Med1 reverses tamoxifen resistance.

Discussion

Breast cancer progression in the presence of tamoxifen is a cardinal manifestation of the development of acquired tamoxifen resistance and represents the principal cause of poor prognosis and death in originally good prognosis hormone-responsive breast tumors. It is of great therapeutic interest to identify the molecular pathways that permit tumor cells to evade therapeutic effects of tamoxifen and continue malignant growth. In this report, we present in vitro and in vivo evidence for a molecular mechanism that contributes to the development of tamoxifen resistance. Our data demonstrate that Med1 is spontaneously upregulated during acquired tamoxifen-resistance development and that tamoxifen-resistant phenotype exhibits persistent activation of ERK and Med1 phosphorylation that has been linked to Med1 coactivation function. Tamoxifen-resistant MCF7-OHT clones exhibit slower growth in response to E2 in culture and in vivo in comparison to MCF7-P cells. Similar to MCF7-P cells, MCF7-OHT clones form ER:Med1 complex in response to E2. We
hypothesize that differential response of MCF7-OHT clones to E2 stimulation is due to the difference between E2-responsive gene profiles among MCF7 and tamoxifen-resistant clones. Different groups of genes may be targeted by ER:Med1 complex in tamoxifen-resistant cells and parental cells in response to E2.

Consistent with a causal role for Med1 in acquired tamoxifen resistance, Med1 knockdown is sufficient to resensitize tamoxifen-resistant cells to tamoxifen-induced growth inhibition and gene expression modulation and to reverse acquired tamoxifen resistance in vivo.

Estrogen-bound ER stimulates transcription of ER-responsive genes utilizing various coactivator complexes (42), whereas tamoxifen-bound ER inhibits transcription via posing a steric impediment inhibiting coactivator recruitment while promoting binding of corepressor complexes (43–45). Importance of coregulators in ER function indicates that alteration in expression and/or activity of coregulatory molecules may potentially alter transcriptional response and downstream biological effects of ER during the development of tamoxifen resistance. Mediator is a conserved multisubunit complex that acts as a functional crossing point between DNA-bound regulatory transcription complexes and basal transcription machinery (46). Overexpression of Med1 in tamoxifen-resistant cells can potentially enhance mediator function leading to altered gene expression in response to tamoxifen. In humans, though Med1 is a critical component of the mediator complex but it

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Fig. 5. Stable knockdown of Med1 reverses tamoxifen resistance in tamoxifen-resistant cells. (A) Immunoblot analysis of Med1 in stable pools of Med1-depleted (Med1shRNA) and vector control (pLKO.1) MCF7-P and MCF7-OHT cells. (B) Clonogenicity of MCF7-P-pLKO.1, MCF7-P-pMed1shRNA, MCF7-OHT-pLKO.1 and MCF7-OHT-pMed1shRNA cells in the presence of 4-OHT (1 μM). *, P < 0.005, compared with untreated controls (MCF7-P-pLKO.1); **, P < 0.001, compared with untreated controls (MCF7-P-pMed1shRNA); #, P < 0.001, compared with untreated controls (MCF7-OHT-pLKO.1); *, P < 0.005, compared with untreated controls (MCF7-OHT-pLKO.1); #, P < 0.001, compared with tamoxifen-treated MCF7-OHT-pLKO.1. (C) Soft agar colony formation in MCF7-P-pLKO.1, MCF7-P-pMed1shRNA, MCF7-OHT-pLKO.1 and MCF7-OHT-pMed1shRNA cells treated with 1 μM of 4-OHT. *, P < 0.005, compared with untreated controls (MCF7-OHT-pLKO.1); #, P < 0.001, compared with tamoxifen-treated MCF7-OHT-pLKO.1. (D) Real-time quantitative PCR analysis of EBA9G, PS2 and IGFl expression in MCF7-P-pLKO.1, MCF7-P-pMed1shRNA, MCF7-OHT-pLKO.1 and MCF7-OHT-pMed1shRNA cells treated with 1 μM of 4-OHT for 2 h. *, P < 0.005, compared with untreated controls (MCF7-P-pLKO.1); #, P < 0.001, compared with untreated controls (MCF7-P-pMed1shRNA); **, P < 0.001, compared with untreated controls (MCF7-OHT-pLKO.1); ***. P < 0.005, compared with untreated controls (MCF7-OHT-pMed1shRNA).
is only variably associated with mediator (47,48). The mechanisms regulating its entry into the core mediator complex involves phosphorylation (49). A novel finding of this study is that tamoxifen-resistant cells have acquired overexpression of Med1 as well as increased phosphorylation of Med1 and ER via importunate ERK activation. Increased phosphorylation of Med1 increases its association with mediator complex hence transactivation functions. Further mechanistic studies will reveal if hyper-phosphorylated ER and Med1 exhibit increased proliferation, in response to tamoxifen. Med1 overexpression also correlated with higher tumor grade in a subset of breast tumors across a panel of breast cancer profiling studies comprising multiple microarray experiments. To further validate our experimental findings, we analyzed Med1 in a large cohort of breast cancer patients from a randomized controlled trial for adjuvant tamoxifen treatment with long-term follow-up. The median follow-up time for patients was 13.9 years. Notably, in the Med1 low subgroup, patients treated with tamoxifen had a significantly better recurrence-free survival compared with control patients in the same subgroup. Multivariate interaction analyses showed that total Med1 was significantly associated with an altered tamoxifen response. As such, based upon our findings, we predict that a large-scale analysis of recurrent human breast cancers after tamoxifen treatment will reveal elevated levels of Med1 compared with tamoxifen-responsive tumors showing no recurrence. ER can get phosphorylated at several amino acid residues encompassing all major structural domains affecting ER ligand binding, dimerization, nuclear localization, DNA binding, coactivator recruitment and transcriptional activation (50). Studies from several groups support the hypothesis that ligand-independent phosphorylation of ER may cause tamoxifen resistance. On one hand, kinases such as ERK support the hypothesis that ligand-independent phosphorylation of ER can get activated in the presence of tamoxifen. Intrinsic to this model is the ERK-directed activation of ER, which has been shown to increase its nuclear translocation, interaction with coregulators, transcriptional activity and receptor turn over (39). Development of tamoxifen resistance attains ERK activation that may directly lead to phosphorylation of Med1 and hence promote its association with mediator complex. Simultaneously, tamoxifen-bound hyperphosphorylated ER may bind to ERE and promote Med1 binding in tamoxifen-resistant cells. In this model, intrinsic abundance of phosphorylated Med1 and ER may potentiate subsequent ER-Med1 complex formation upon tamoxifen stimulus so that a maximal transcription response is rapidly achieved and sustained. Future studies should reveal whether other cellular signal transduction pathways target Med1 and ER for regulatory phosphorylation and affect their functional roles in acquired tamoxifen resistance.

Importantly, the association that we detected between high Med1 expression in a subset of breast tumors and recurrence after tamoxifen treatment in humans implies that Med1 overexpression may play a role in promoting tamoxifen resistance. Consistent with this, we have demonstrated that Med1 knockdown is sufficient to reverse tamoxifen resistance in mice tumor studies. We also found that Med1 overexpression was associated with increased aggressiveness and higher clinical risk in human tumor studies. It is worth noting that the association between Med1 overexpression and aggressiveness is consistent with our observation that mice tumors exhibiting intact Med1 overexpression levels showed higher Ki-67 staining, indicative of increased proliferation, in response to tamoxifen. Med1 overexpression also correlated with higher tumor grade in a subset of breast tumors across a panel of breast cancer profiling studies comprising multiple microarray experiments. To further validate our experimental findings, we analyzed Med1 in a large cohort of breast cancer patients from a randomized controlled trial for adjuvant tamoxifen treatment with long-term follow-up. The median follow-up time for patients was 13.9 years. Notably, in the Med1 low subgroup, patients treated with tamoxifen had a significantly better recurrence-free survival compared with control patients in the same subgroup. Multivariate interaction analyses showed that total Med1 was significantly associated with an altered tamoxifen response. As such, based upon our findings, we predict that a large-scale analysis of recurrent human breast cancers after tamoxifen treatment will reveal elevated levels of Med1 compared with tamoxifen-responsive tumors showing no recurrence. ER can get phosphorylated at several amino acid residues encompassing all major structural domains affecting ER ligand binding, dimerization, nuclear localization, DNA binding, coactivator recruitment and transcriptional activation (50). Studies from several groups support the hypothesis that ligand-independent phosphorylation of ER may cause tamoxifen resistance. On one hand, kinases such as ERK support the hypothesis that ligand-independent phosphorylation of ER can get activated in the presence of tamoxifen. Intrinsic to this model is the ERK-directed activation of ER, which has been shown to increase its nuclear translocation, interaction with coregulators, transcriptional activity and receptor turn over (39). Development of tamoxifen resistance attains ERK activation that may directly lead to phosphorylation of Med1 and hence promote its association with mediator complex. Simultaneously, tamoxifen-bound hyperphosphorylated ER may bind to ERE and promote Med1 binding in tamoxifen-resistant cells. In this model, intrinsic abundance of phosphorylated Med1 and ER may potentiate subsequent ER-Med1 complex formation upon tamoxifen stimulus so that a maximal transcription response is rapidly achieved and sustained. Future studies should reveal whether other cellular signal transduction pathways target Med1 and ER for regulatory phosphorylation and affect their functional roles in acquired tamoxifen resistance.

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prognosis and de novo endocrine resistance. Future studies utilizing clinical samples relevant for acquired tamoxifen resistance might show tighter associations between specific ER phosphorylation and development of acquired resistance.

For many years, oncologists have used ER/PR clinically to select treatment options. In spite of this, 25% of ER+/PR+ tumors, 66% of ER+/PR– tumors and 55% of ER–/PR+ tumors either present de novo resistance or develop acquired resistance (3). To date, there are no dependable markers to identify patients who are at risk for tumor recurrence in the setting of adjuvant tamoxifen. The ability to predict the development of tamoxifen resistance in hormone-positive tumors can be extremely useful in facilitating the use of alternative hormonal therapies such as the aromatase inhibitors letrozole and anastrozole (58–60), chemotherapeutic agents (61) or inhibitors of other signaling pathways (62–64) in this subset of patients hence improving their clinical outcome. Our results suggest the utility of Med1 expression levels in identifying subset of patients who are at risk for tumor recurrence in the setting of tamoxifen therapy and who may benefit from alternative therapeutic approaches. Beyond the ability to predict tamoxifen resistance, our findings raise the important possibility that acquired tamoxifen resistance might be prevented by therapeutic agents that inhibit Med1, either by preventing its upregulation or by inhibiting its function. For example, inhibition of Med1 phosphorylation by ERK inhibition inhibits transactivation function of Med1. Hence, development of cancer therapeutics that targets these upstream pathways may be useful.
Future studies utilizing small molecule inhibitors for ERK activation are required to show if ERK inhibition is sufficient to reverse tamoxifen resistance \textit{in vivo}. A definite causal role of Med1 cannot be yet confirmed in human breast tumor tamoxifen resistance since no specific drugs are available to specifically target Med1. Nonetheless, our findings may potentially open new avenues of research into the role of Med1 in the prediction and therapeutic targeting of acquired tamoxifen resistance.
Med1 mediates tamoxifen resistance in breast cancer

Supplementary material
Supplementary Materials, Figures S1–S5 and Table 1 can be found at http://carcin.oxfordjournals.org/.

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