Combined p53- and PTEN-deficiency activates expression of mesenchyme homeobox 1 (MEOX1) required for growth of triple-negative breast cancer

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Triple-negative breast cancer (TNBC) is an aggressive cancer subtype for which effective therapies are unavailable. TNBC has a high frequency of tumor protein p53 (Tp53/p53)- and phosphatase and tensin homolog (PTEN) deficiencies, and combined p53- and PTEN-deficiency is associated with poor prognosis and poor response to anticancer therapies. In this study, we discovered that combined p53- and PTEN-deficiency in TNBC activates expression of the transcription factor mesenchyme homeobox 1 (MEOX1). We found that MEOX1 is expressed only in TNBC cells with frequent deficiencies in p53 and PTEN, and that its expression is undetectable in luminal A, luminal B, and HER2+ subtypes, as well as in normal breast cells with wild-type (WT) p53 and PTEN. Notably, siRNA knockdown of both p53 and PTEN activated MEOX1 expression in breast cancer cells, whereas individual knockdowns of either p53 or PTEN had only minimal effects on MEOX1 expression. MEOX1 knockdown abolished cell proliferation of p53- and PTEN-deficient TNBC in vitro and inhibited tumor growth in vivo, but had no effect on the proliferation of luminal and HER2+ cancer cells and normal breast cells. RNA-Seq and immunoblotting analyses showed that MEOX1 knockdown decreased expression of tyrosine kinase 2 (TORK2), signal transducer and activator of transcription 5B (STAT5B), and STAT6 in p53- and PTEN-deficient TNBC cells. These results reveal the effects of combined p53- and PTEN-deficiency on MEOX1 expression and TNBC cell proliferation, suggesting that MEOX1 may serve as a potential therapeutic target for managing p53- and PTEN-deficient TNBC.

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype that lacks effective therapy. Among four subtypes of breast cancers (luminal A, ER+/PR+/HER2--; luminal B, ER+/PR+/HER2 low; HER2+, ER−/PR−/HER2++; TNBC, ER−/PR−/HER2−), TNBC accounts for 10–20% of all breast cancers (1–5). TNBC has high rates of metastasis, and metastatic TNBC patients have poor 5-year survival (30%) (5, 6). Although tremendous advances have been made for therapies against the other three subtypes of breast cancers (7–14), TNBC still lacks effective treatment options.

TNBC has high frequencies of tumor protein p53 (Tp53/p53) and phosphatase and tensin homolog (PTEN) wild-type (WT) deficiency (9, 15–23). To ascertain new therapeutic alternatives, researchers have set out to explore the complex heterogeneity of TNBC tumors and its evolving biology; in doing so studies demonstrate frequent genetic aberrations in tumor suppressor genes of p53 and PTEN as critical “driver mutations” conferring a selective advantage for TNBC aggressive tumorigenesis, drug resistance, and poor prognosis (9, 15, 19–23). p53 deficiency is observed in ~80% of TNBC tumors (15, 16). These include loss-/gain-of-function mutations (16, 24, 25) or loss of expression of p53 by epigenetic alteration of p53, which increases tumor formation and metastasis (26–29). PTEN deficiency is observed in 35% of TNBC tumors (15). These include a small percentage of PTEN mutations (2–9%) and the majority of cases with reduced or loss of expression (30–32), which is due to loss of heterozygosity (~40%) and aberrant promoter epigenetic hypermethylation (~30–50%) (30, 33, 34). Heterozygous PTEN+/− mice (61–83%) develop basal-like breast cancer (35–37).

Combined p53- and PTEN-deficiency is seen in 20–30% of TNBC, which is associated with poor prognosis and survival (19, 23). Analysis of human TNBC tumors show patients with low p53 and PTEN expressions display poorer prognosis with worse metastatic free survival relative to patients with normal p53 and PTEN levels (19). In addition, p53 and PTEN deletion in normal mammary epithelia of mice induces the formation of TNBC tumors (19, 23). These tumors manifest fast and aggressive TNBC hallmark characteristics with increased metastasis (19, 23). Furthermore, mice with loss of both p53 and PTEN in normal mammary epithelia exhibit decreased tumor free survival relative to loss of either p53 or PTEN alone (19, 23). In normal breast cells (MCF-10A), combined p53 and PTEN knockdown transforms these normal breast cells to aggressive metastatic cancer cells in vitro and in mice (38).

Combined p53- and PTEN-deficiency in TNBC is associated with poor responses to various therapies. Chemotherapy is the first line of treatment for TNBC. However, molecular profiles of TNBC residual disease unresponsive to neoadjuvant chemotherapy show high frequencies of genetic aberrations of both p53 and PTEN (9). Although PTEN-deficiency activates the PI3K/AKT/mTOR signaling pathway that can be inhibited by respective inhibitors to show promising efficacy in some breast cancers (39, 40), clinical evaluation of the pathway inhibitors (such as Everolimus and Ipatasertib) achieves limited success in TNBC patients (41–43). Combined p53- and PTEN-deficiency...
in TNBC may result in a unique molecular signaling pathway, which may be distinct from the deficiency of p53 or PTEN alone as well as p53 and PTEN WT.

In this paper, we intended to identify molecular targets in TNBC with combined p53- and PTEN-deficiency, as well as offer insight into its poorly understood molecular biology. Our data show that combined p53- and PTEN-deficiency in TNBC activates a specific molecular target mesenchyme homeobox 1 (MEOX1), which may serve as a therapeutic target for p53- and PTEN-deficient TNBC. MEOX1 expression was surveyed in four subtypes of breast cancer cell lines, which show it is only expressed in TNBC cells with frequent p53- and PTEN-deficiencies, but undetectable in luminal A, luminal B, and HER2+ breast cancer subtypes, as well as in normal breast cells with WT p53 and PTEN. Single and double siRNA knockdowns of p53 and PTEN were used to investigate how they regulate MEOX1 expression in breast cancer cells. Furthermore, we studied the function of MEOX1 in p53- and PTEN-deficient TNBC in regulating cell proliferation and invasion in vitro and tumor growth in vivo. Finally, we also explored the downstream targets of MEOX1 in TNBC cells. These data suggest that MEOX1 may serve as a potential therapeutic target for p53- and PTEN-deficient TNBC.

Results

TNBC p53- and PTEN-deficiency is associated with MEOX1 expression

We examined MEOX1 expression by RT-qPCR and found that MEOX1 is only expressed in TNBC cells with frequent p53- and PTEN-deficiencies, but undetectable in normal breast cells of MCF-10A and three other breast cancer subtypes (Fig. 1A). Data shows TNBC cell lines of MDA-MB-231, BT-549, SUM149, MDA-MB-468, SUM159, and MDA-MB-453 have high expression of MEOX1; as expected, these in vitro TNBC cell lines show high incidences of p53 and PTEN genetic aberrations conferring loss of WT function for both tumor-suppressor genes (Table 1) (36, 44–52). In contrast, results show no expression of MEOX1 in the luminal subtypes of MCF-7, T-47D, and ZR-75-1. Similarly, no expression of MEOX1 is seen in the HER2+ subtypes of BT-474, MDA-MB-361, and ZR-75-30.

Combined p53- and PTEN-deficiency activates MEOX1 expression compared with WT and single deficiency of p53 or PTEN

To confirm if p53 and PTEN regulates MEOX1 expression, we used siRNA to knockdown p53 and/or PTEN. The knockdown efficiency of p53 and PTEN was confirmed using RT-qPCR (Fig. S1). The data show that siRNA knockdown of both p53 and PTEN in normal immortalized MCF-10A, luminal MCF-7, and triple-negative SUM159 increases MEOX1 expression (Fig. 1, B–D), whereas siRNA knockdown of either p53 or PTEN alone only have minor effects on MEOX1 expression in all three different breast cancer subtypes.

It is important to note that normal immortalized MCF-10A and luminal MCF-7 cells are used because both have normal WT function and expression of p53 and PTEN (Table 1) (36, 44–52). However, triple-negative SUM159 cells harbor loss of WT function for p53 with normal function of PTEN (Table 1) (36, 44–52). Finding a triple-negative cell line with no genetic aberrations in p53 is challenging, as such SUM159 cells are just used as proof of concept. Nevertheless, data clearly shows the expression of MEOX1 is specific to TNBC and negatively regulated by both tumor suppressor genes of p53 and PTEN.

Interestingly, HER2 also negatively regulates MEOX1 expression. In luminal MCF-7, stable knockdown of PTEN alone increased MEOX1 expression, however, stable overexpression of HER2 down-regulated its expression (Fig. S2A). Furthermore, HER2 expression inhibited MEOX1 expression when PTEN was knocked down in MCF-7 cells. Similar results were observed in triple-negative SUM159 cells. Stable knockdown of PTEN alone in SUM159 increased the expression of MEOX1, but stable overexpression of HER2 down-regulated its expression (Fig. S2B). As seen with MCF-7, overexpression of HER2 inhibited MEOX1 expression when PTEN was concurrently knocked down in SUM159. This point was further established when performing HER2, p53, and PTEN siRNA experiments in the HER2+ breast cancer cell line BT-474. Knockdown of p53 or PTEN alone had no effect on MEOX1 expression in BT-474, and neither did dual knockdown of both p53 and PTEN, where HER2 levels are high in BT-474 (Fig. S2C). Only during dual knockdown of p53 with PTEN or HER2 with PTEN did expression of MEOX1 start to increase. However, the most significant increase of MEOX1 expression was seen when all three genes of HER2, p53, and PTEN were down-regulated.

MEOX1 knockdown decreases in vitro cell proliferation of p53- and PTEN-deficient TNBC

To assess MEOX1’s function on cell proliferation, we used three different siRNA treatments to knockdown MEOX1 in p53- and PTEN-deficient TNBC (claudin-low BT-549 and basal-like MDA-MB-468). These two different intrinsic subtypes of TNBC harbor inherent genetic aberrations in p53 and PTEN conferring loss of WT function for both tumor-suppressor genes (Table 1) (36, 44–52). The siRNA knockdown efficiency of MEOX1 was confirmed using RT-qPCR (Fig. S3). The data show that knocking down MEOX1 using two single siRNAs and a pooled mixture of four different siRNAs significantly decrease cell proliferation of these two TNBC cell lines (Fig. 2, A and B). However, two single siRNAs and a pooled mixture of four different siRNAs show no significant change in cell proliferation in normal immortalized MCF-10A, luminal ZR-75-1, and HER2+ BT-474 (Fig. 2, C–E). These data suggest that MEOX1 expression may be critical and specific to regulating cell proliferation in p53- and PTEN-deficient TNBC. In addition, the siRNA experiments in MCF-10A, ZR-75-1 and BT-474 also suggest that these siRNAs of MEOX1 have no off-target effects on these cell lines, which have undetectable levels of MEOX1. These data may also suggest these cells do not rely on MEOX1 for their proliferation.
Knockdown of MEOX1 decreases in vitro cell self-renewal of p53- and PTEN-deficient TNBC

To evaluate if MEOX1 also regulates cell self-renewal of p53- and PTEN-deficient TNBC, we used 3D mammosphere formation efficiency for consecutive passages in vitro with three different MEOX1 siRNA knockdown treatments previously described (53). Mammosphere formation assays show knocking down MEOX1 in both claudin-low BT-549 and basal-like MDA-MB-468 decreases both primary and secondary consecutive mammosphere formations (Fig. 3, A–D and F). As such, MEOX1 knockdown decreases cell self-renewal function of p53- and PTEN-deficient TNBC. In contrast, stable overexpression of MEOX1 in normal immortalized MCF-10A increases mammosphere formation, regulating an increase in cell self-renewal (Fig. 3, E and G). The overexpression efficiency of MEOX1 in MCF-10A was confirmed using RT-qPCR (Fig. S4).

MEOX1 knockdown decreases in vitro cell migration and invasion of p53- and PTEN-deficient TNBC

Cell directional migration and invasion through an extracellular matrix are important properties of metastasis (54–56). In vitro cell migration and invasion assays were conducted to ascertain the role of MEOX1 as a metastatic regulator of p53- and PTEN-deficient TNBC. Knocking down MEOX1 in both claudin-low BT-549 and basal-like MDA-MB-468 decreases both migration and invasion of TNBC cells (Fig. 4, A, C, D, and F). Furthermore, the invasion index decreases by ~50% in claudin-
low BT-549 and ~30% in basal-like MDA-MB-468 cells (Fig. 4, B and E).

It is worth noting that after 72 h of siRNA transfection, cells were trypsinized and counted three times to make certain only live viable cells were plated for migration and Matrigel invasion assays. In addition, these live cells plated were serum starved to establish cell synchronization, minimizing any potential differences in cell migration and invasion that may be seen due to varying cell proliferation rates and serum components. Furthermore, we also calculated the invasion index, which normalized the ratio of the cells invading through Matrigel insert to cells migrating through control insert (\( \text{Invasion index} = \frac{\text{mean # of cells migrating through control insert}}{\text{mean # of cells migrating through Matrigel insert}} \)). Invasion Index is % invasion in MEOX1 siRNA treatment/% invasion in control siRNA treatment. These results indicate MEOX1 regulates metastatic properties of cell migration and invasion in p53- and PTEN-deficient TNBC.

**Inducible knockdown of MEOX1 inhibits in vivo tumor growth of p53- and PTEN-deficient TNBC**

To evaluate MEOX1 function on tumor growth in vivo, we used a doxycycline-inducible MEOX1 shRNA knockdown system in basal-like TNBC cells MDA-MB-468 with p53- and PTEN-deficiency. The inducible knockdown efficiency of MEOX1 by doxycycline was confirmed using RT-qPCR (Fig. S5). Using NOD/SCID mice, we used two treatment regimens (adjuvant and advanced treatment) to test MEOX1 function on xenograft tumor growth in orthotopic mammary fat pads. In an adjuvant regimen, MEOX1 shRNA knockdown was induced with administration of doxycycline 3 days following surgery. In an advanced treatment regimen setting, MEOX1 shRNA knockdown was induced with administration of doxycycline after palpable or visible tumor growth. Data shows that regardless of when doxycycline was administered to induce shRNA, in an adjuvant or advanced treatment regimen, MEOX1 knockdown decreases in vivo tumor growth of p53- and PTEN-deficient TNBC (Fig. 5, A and B). However, knockdown of MEOX1 seems to have a greater impact on decreasing tumor growth in an adjuvant setting.

**MEOX1 regulates JAK/STAT signaling in p53- and PTEN-deficient TNBC**

We used RNA-seq to ascertain mechanisms involved in MEOX1 functional regulation and potential downstream targets of p53- and PTEN-deficient TNBC. IPA was used to assess how RNA-seq differential gene expression between MEOX1 knockdown versus negative control effects biological signaling pathways in p53- and PTEN-deficient TNBCs. RNA-seq IPA comparison and individual analyses of p53- and PTEN-deficient claudin-low BT-549 and basal-like MDA-MB-468 show important biological signaling pathways are perturbed following MEOX1 knockdown (Fig. 6, A–C). This includes well-known JAK/STATs, MAPK, p70S6K, CXCR4, actin cytoskeleton signaling, integrin signaling, Goq, RhoA, and Rho family of GTPases. These pathways offer insight to potential mechanistic transductions involved in MEOX1 regulation of proliferation and metastasis in these p53- and PTEN-deficient TNBC cells.

In a comparison analysis or individual analysis of p53- and PTEN-deficient TNBCs signaling of the JAK/STAT pathway as a whole is disrupted (Fig. S6). Although inactivation of JAK/STAT signaling has a significantly high \( p \) value of 0.0000089 in BT-549 and a close to significant \( p \) value of 0.060 in MDA-MB-468, inactivation of the STAT3 signaling pathway has significant \( p \) values < 0.05 and z-scores < −2 in both cell lines. (Table S1). To confirm these findings, we performed Western blotting analysis for JAK/STAT pathways in MEOX1 siRNA knocked down cells in two TNBC cell lines. The data show that knockdown of MEOX1 decreases JAK1 and TYK2 protein levels in claudin-low BT-549, but only decreases TYK2 protein levels in basal-like MDA-MB-468 (Fig. 7, A and B). Analyzing the STAT family of proteins shows an increase in STAT1 as well as decreases in STAT5B and STAT6 protein levels in claudin-low BT-549 (Fig. 7A). Similarly, decreases in STAT5B and STAT6 protein levels are also observed in basal-like MDA-MB-468 (Fig. 7B). Further analysis of phosphorylated STATs shows a decrease of P-STAT3 (Tyr-705) in claudin-low BT-549, however, no change in phosphorylated STATs are seen in basal-like MDA-MB-468 cells (Fig. 7, A and B). In analyzing inhibitory JAK/STAT signaling pathways, which were reported to be associated with p53 and PTEN (38), no significant changes by
siRNA knockdown of MEOX1 were found in PIAS1, PIAS3, PIAS4, SOCS2, and SOCS3 (Fig. S7).

In summary, Western blotting analysis for the JAK/STAT pathway shows that MEOX1 knockdown has a significant impact on decreasing JAK1, TYK2, STAT5B, STAT6, and P-STAT3 (Tyr-705) protein levels in claudin-low BT-549, but only has a significant impact on decreasing TYK2, STAT5B, and STAT6 protein levels in basal-like MDA-MB-468. Interestingly, STAT1 protein levels increase in claudin-low BT-549 after MEOX1 knockdown.

Discussion

In this article, insight is offered into the complex and poorly understood molecular biology of p53- and PTEN-deficient TNBC by studying the functional and mechanistic role of MEOX1, presenting a specific therapeutic target. A homeobox transcription factor, MEOX1 is well known for its role in somitogenesis of the developing vertebrate embryo (57–61). Although mainly expressed and studied in the context of development, recent research demonstrates a link between MEOX1 and its activation in cancer. Thiaville and colleagues (62) show evidence involving MEOX1 in ovarian cancer, describing MEOX1 as an important pre-B-cell leukemia homeobox-1 (PBX1) cofactor and target gene for tumor cell growth. Additional research links the function of MEOX1 to increased breast cancer cell proliferation and correlates nuclear expression of MEOX1 with poor overall breast cancer patient survival, as well as increased lymph node metastasis and high tumor stage (43).

In investigating the role of homeobox transcription factor MEOX1 in breast cancer, RNA expression analysis of in vitro breast cancer cell lines show MEOX1 is up-regulated in TNBC and no expression of MEOX1 is seen in luminal or HER2+ subtypes. Furthermore, RNA expression analysis also show that MEOX1 is negatively regulated by both tumor-suppressor genes of p53 and PTEN. In vitro p53 and PTEN siRNA knockdown experiments demonstrate that only with combined loss of p53 and PTEN does expression of MEOX1 significantly increase. High expression of MEOX1 in p53- and PTEN-deficient TNBC has a crucial role for regulating cell proliferation and cell self-renewal. Experiments with siRNA knockdown show a decrease in MEOX1 expression significantly decreases in vitro cell proliferation and cell self-renewal of two different intrinsic subtypes of claudin-low and basal-like p53- and PTEN-deficient TNBC. Additionally, no effect on cell proliferation is seen in normal immortalized, luminal, or HER2+ breast cancer cells upon knockdown of the transcription factor; these data suggest that targeting MEOX1 can specifically decrease the aggressive proliferative behavior of p53- and PTEN-deficient TNBC with minimal potential of off-target effects. In vivo experiments corroborate in vitro results, where tumor xenograft mouse models using doxycycline-inducible shRNA show knockdown of MEOX1 decreases tumor growth of basal-like p53- and PTEN-deficient TNBC in an adjuvant and advanced

Figure 2. MEOX1 knockdown specifically decreases in vitro cell proliferation of p53- and PTEN-deficient TNBC. A and B, using three different siRNA treatments, MEOX1 knockdown in triple-negative cells (claudin-low BT-549 and basal-like MDA-MB-468) significantly decreases cell proliferation of these p53- and PTEN-deficient TNBCs. C-E, using three different siRNA treatments, MEOX1 knockdown in normal immortalized MCF-10A, luminal ZR-75-1, and HER2+ BT-474 shows no significant change in cell proliferation. Results are shown as mean ± S.D., n = 6, two-way ANOVA statistical analysis with Dunnett’s multiple comparisons test, ****, p < 0.0001.
Figure 3. Knockdown of MEOX1 decreases in vitro cell self-renewal of p53- and PTEN-deficient TNBC. A–D and F, using different siRNA treatments, a decrease in MEOX1 expression decreases primary and secondary consecutive mammosphere formations in triple-negative cells (claudin-low BT-549 and basal-like MDA-MB-468). As such, knockdown of MEOX1 decreases self-renewal properties of p53- and PTEN-deficient TNBCs. E and G, normal immortalized MCF-10A cells with stable overexpression of MEOX1 increases mammosphere formation, regulating an increase in cell self-renewal. Results are shown as mean ± S.D., one-way ANOVA statistical analysis with Dunnnett’s multiple comparisons test was used for A–D, unpaired t test statistical analysis for E, **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001.
treatment regimen. However, a greater inhibition of tumor growth is seen in an adjuvant regimen. As such, MEOX1 targets particularly in an adjuvant setting can benefit to decrease the rapid proliferative property of TNBC tumors with p53- and PTEN-deficiency. Moreover, results also show that MEOX1 regulates metastasis; knockdown experiments using siRNA for MEOX1 decreases metastatic functions of migration and invasion in both claudin-low and basal-like intrinsic subtypes. Thus, along with decreasing rapid cell proliferation, targeting MEOX1 can also have a crucial role in reducing the aggressive metastatic potential of p53- and PTEN-deficient TNBC.

To ascertain the mechanistic biological signaling pathways involved in MEOX1 function of regulating cell proliferation and metastasis in p53- and PTEN-deficient TNBC, RNA-seq was utilized. RNA-seq mechanistic investigation of IPA comparison and individual analyses of both claudin-low and basal-like p53- and PTEN-deficient TNBC shows JAK/STAT signaling pathways are inactivated upon MEOX1 knockdown. Indeed, investigation of the JAK/STAT pathway using Western blotting analysis show MEOX1 knockdown increases STAT1 but decreases JAK1, TYK2, STAT5B, STAT6, and P-STAT3 (Tyr-705) protein levels in claudin-low, as well as decreases TYK2, STAT5B, and STAT6 protein levels in basal-like p53- and PTEN-deficient TNBC.

Although still undergoing extensive research and scrutiny, the JAK family proteins of JAK1 and TYK2, as well as STAT family proteins of STAT1, STAT3, STAT5, and STAT6 have been implicated in multiple cancers, including breast tumorigenic pathways of proliferation and metastasis (63–70). Research shows aberrations in JAK/STAT signaling promote oncogenic properties of proliferation and metastasis, such as increased cell growth, migration, and invasion (63–65). In addition, research has also previously shown a link between aberrations in JAK/STAT signaling with loss of p53 and PTEN WT function in TNBC cells (38).
Combined p53- and PTEN-deficiency activates MEOX1 in TNBC

Figure 5. Inducible knockdown of MEOX1 inhibits in vivo tumor growth of p53- and PTEN-deficient TNBC. A, in an adjuvant setting, MEOX1 shRNA knockdown was induced with doxycycline 3 days after orthotopic mammary fat pad injections. B, in an advanced setting, MEOX1 shRNA knockdown was induced with doxycycline after palpable or visible tumor growth following orthotopic mammary fat pad injections. In both settings, knocking down MEOX1 decreases growth of basal-like MDA-MB-468 p53- and PTEN-deficient TNBC xenograft tumors in NOD/SCID mice. However, a greater impact on decreasing tumor growth with MEOX1 knockdown is seen in the adjuvant setting. Results are shown as mean ± S.D., n = 5, two-way ANOVA statistical analysis with Dunnett’s multiple comparisons test, ****, p ≤ 0.0001.

Kim and colleagues (38) demonstrate in vitro stable knockdown of p53 and PTEN in normal immortalized MCF-10A transforms cells to resemble basal-like and claudin-low TNBCs. These cells display increased activation of the IL6-STAT3-NFκB signaling pathway through proteolytic degradation of SOCS3, as well as manifest aggressive proliferative metastatic tumors following xenograft orthotopic mammary fat pad injections in NOD/SCID mice. However, a greater impact on decreasing tumor growth with MEOX1 knockdown is seen in the adjuvant setting. Results are shown as mean ± S.D., n = 5, two-way ANOVA statistical analysis with Dunnett’s multiple comparisons test, ****, p ≤ 0.0001.

It is worth noting that the expression of MEOX1 was detected by its RNA levels using only RT-qPCR in this study. No antibody for MEOX1 was found to be sensitive and specific enough to confirm its protein levels in various cells and confirm siRNA or shRNA knockdown. In addition, while the siRNAs and shRNAs showed similar knockdown efficiency for MEOX1 RNA, it is not known if these knockdown treatments will have similar efficiency in down-regulating MEOX1 protein levels because knockdown of RNA and protein are not always consistent (71). These discrepancies need to be further investigated in the future.

Establishing effective therapeutic targets will require analyzing the interplay of signaling pathways transformed by multiple combined genetic aberrations, which ultimately govern TNBC tumorigenesis and heterogeneity. Studying MEOX1 offers biological insight and presents an opportunity for specified targeting of combined p53- and PTEN-deficient TNBC, which lacks actionable targets. Given the functional role of MEOX1 to decrease tumorigenic properties of proliferation and metastasis, targeting MEOX1 can help decrease the aggressive proliferative and metastatic potential of p53- and PTEN-deficient TNBC. Since genetic aberrations in tumor-suppressor genes of p53 and PTEN are frequent common driver mutations for tumorigenesis in TNBC, targeting MEOX1 may provide a potential therapeutic strategy. Given that MEOX1 is expressed during development and not known to be present in adult tissues, therapies utilizing the latest treatment modalities in the clinic to target MEOX1 are suspected to be specific in p53- and PTEN-deficient TNBC.

Materials and Methods

In vitro cell culture growth conditions

Gibco Life Technologies base media was used to grow in vitro breast cancer cell lines and ATCC recommended media formulation guidelines were followed. MCF-10A cells were grown in DMEM/F-12 supplemented with antibiotic-antimycotic, 5% horse serum, 20 ng/ml of epidermal growth factor (EGF), 100 ng/ml of cholera toxin, 500 ng/ml of hydrocortisone, and 10 μg/ml of insulin. MCF-7 cells were grown in Earle’s minimal essential medium supplemented with antibiotic-antimycotic, 10% fetal bovine serum, and 1 mM sodium pyruvate. T-47D cells were grown in RPMI supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 5 μg/ml of insulin, and 1 mM sodium pyruvate. ZR-75-1, BT-474, and ZR-75-30 cells were grown in RPMI supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 1 mM sodium pyruvate, and 10 mM HEPES. MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were grown in DMEM supplemented with antibiotic-antimycotic, 10% fetal bovine serum, and 1 mM sodium pyruvate. MDA-MB-361 cells were grown in DMEM supplemented with antibiotic-antimycotic, 20% fetal bovine serum, and 1 mM sodium pyruvate. MDA-M-B-361 cells were grown in DMEM supplemented with antibiotic-antimycotic, 10% fetal bovine serum, 1 mM sodium pyruvate, and 10 mM HEPES. SUM149 and SUM159 cells were grown in Ham’s F-12 supplemented with antibiotic-antimycotic, 5% fetal bovine serum, 1 μg/ml of hydrocortisone, 5 μg/ml of insulin, and 10 mM HEPES. All cells were grown in a humidified 37 °C incubator with 5% CO2.

In vitro cell culture transient transfection for siRNA knockdown

Each breast cancer cell line was plated onto 6-well–plates at ~50% confluence per well. For each well, 2 × 105 cells of BT-549, MDA-MB-468, SUM159, MCF-10A; 6 × 105 cells of ZR...
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**A**

### Canonical Pathways
- Actin Cytoskeleton Signaling
- RhoA Signaling
- STAT3 Pathway
- α-Adrenergic Signaling
- Integrin Signaling
- Apoptosis Signaling
- Prolactin Signaling
- p53 Signaling
- UVA-Induced MAPK Signaling
- Signaling by Rho Family GTPases
- Chemokine Signaling
- Wnt/β-catenin Signaling
- p70S6K Signaling
- Interferon Signaling
- Thrombin Signaling
- GNRH Signaling
- NF-κB Signaling
- CXCR4 Signaling
- Goq Signaling

**B**

**BT-549**

**C**

**MDA-MB-468**

### Canonical Pathways
- NGF Signaling
- JAK/Stat Signaling
- Endothelin-1 Signaling
- Thrombopoietin Signaling
- SAPK/JNK Signaling
- IL–3 Signaling
- HGF Signaling
- TNFR1 Signaling
- iNOS Signaling
- Renin–Angiotensin Signaling
- LXR/RXR Activation
- 14–3–3–mediated Signaling
- TNFR2 Signaling
- Growth Hormone Signaling
- Insulin Receptor Signaling
- PPARα/RXRα Activation
- ILK Signaling
- ERK/MAPK Signaling
- PPAR Signaling

**Ratio**

\[ \text{Ratio} = \frac{\text{# genes in RNA-seq data associated with pathway}}{\text{# of genes in reference pathway}} \]
Combined p53- and PTEN-deficiency activates MEOX1 in TNBC

Figure 6. MEOX1 knockdown perturbs important canonical biological signaling pathways in p53- and PTEN-deficient TNBC. A, individual IPA assessment of MEOX1 knockdown in p53- and PTEN-deficient claudin-low BT-549 cells of basal-like MDA-MB-468 shows activation and inactivation of canonical signaling pathways. B, individual IPA assessment of MEOX1 knockdown in p53- and PTEN-deficient basal-like MDA-MB-468 shows inactivation of canonical signaling pathways. JAK/STAT signaling pathways are inactivated in both TNBC cell lines.

Figure 7. MEOX1 regulates JAK/STAT signaling in p53- and PTEN-deficient TNBC. A, knockdown of MEOX1 in p53- and PTEN-deficient claudin-low BT-549 decreases JAK1, TYK2, STAT5B, and STAT6 protein levels; however, an increase in STAT1 protein levels are observed. P-STAT3 (Tyr-705) protein levels, however, an increase in STAT1 protein levels.

75-1; and 9 \times 10^5 cells of BT-474 were required to achieve 50% confluence. After 24 h of plating, cells were transfected with siRNA using Invitrogen Lipofectamine RNAiMAX Reagent (No. 13778-150); transfection was conducted according to the manufacturer’s instructions and antibiotic-antimycotic was omitted from the media to increase transfection efficiency as well avoid unnecessary toxicity. For MEOX1 knockdown experiments, 50 nM Negative Control siRNA and 50 nM MEOX1 siRNA for each treatment group was utilized.

All siRNAs used to conduct experiments were purchased from Qiagen. Negative Control siRNA (No. 1027281) contained target sequence, 5'-CAGGGTATCGAGGATTACAA-3'; sense strand, 5'-GGGUAUCGACGAAUCAAAUU-3'; and antisense strand, 5'-UUUGUAUCGUGCAUACCCCU-3'. Three different MEOX1 siRNA treatments were used to validate results. MEOX1 siRNA 1 (No. SI00630266) contained target sequence, 5'-CAGCTTTGACTGGGTGACAA-3'; sense strand, 5'-GGCGUAUACAGGAAUATT-3'; and antisense strand, 5'-UUUGUAUACCGCAUACGCTT-3'. MEOX1 siRNA 2 (No. SI00630280) contained target sequence, 5'-AAGCTAATTGTGCCAGCTCAA-3'; sense strand, 5'-GCUAAUUGUGCGACAAATT-3'; and antisense strand, 5'-UUGAGCUCCGCAAUUGCCTT-3'. MEOX1 siRNA Mixture is a pool of four different MEOX1 siRNAs mixed together for a final concentration of 50 nM, matching the concentration of MEOX1 siRNA 1 and MEOX1 siRNA 2. This is a technique commonly carried out using different siRNAs each at low concentrations to ensure there are no off-target effects that may be caused when using one siRNA alone. The MEOX1 siRNA mixture pools together 12.5 nM of each MEOX1 siRNA 1, MEOX1 siRNA 2, and MEOX1 siRNA 3 (No. SI03145205) with target sequence, 5'-AGCTGGCGACTGGGTGGACAA-3'; sense strand, 5'-CUGCCGACUGGAAAAGUATT-3'; and antisense strand, 5'-UUACUUUCCAGGACUGCTT-3', as well as MEOX1 siRNA 4 (No. SI04293310) with target sequence, 5'-TCCACGATTTCTGGATGAA-3'; sense strand, 5'-CACGAUUACUGGAUUGAAATT-3'; and antisense strand, 5'-UUUCAAUCCGAGAUCUGGGA-3'.

To knockdown HER2, Qiagen FlexiTube GeneSolution (No. GS2064) was tested. Functionally verified HER2 siRNA (No. SI02223571) validated by Qiagen to knockdown HER2 contained target sequence, 5'-AACAAAGAATCTTAGCAGA-3'; sense strand, 5'-CAAGAAGAAUUCUGACGAA-3'; and antisense strand, 5'-UUCGUCUAGAUAUUUGTT-3'. To knockdown p53, Qiagen FlexiTube GeneSolution (No. GS7157) was tested. Functionally verified p53 siRNA (No. SI02655170) validated by Qiagen to knockdown p53 contained target sequence, 5'-AAGGGAATTCTGGTGGAGT-3'; sense strand, 5'-GGAAUUGUGCGUGGAGUTT-3'; and antisense strand, 5'-ACUCCACCGAAUUUCCTT-3'.

To knockdown PTEN, Qiagen FlexiTube GeneSolution (No. GS5728) was tested. Functionally verified PTEN siRNA (No. SI00301504) validated by Qiagen to knockdown PTEN contained target sequence, 5'-AAGGCGTATACAGGAAACT-3'; sense strand, 5'-GGCGUAUACAGGAAACAAAT-3'; and antisense strand, 5'-UUUGUUGCGUAUACCGGCTT-3'.
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**In vitro cell culture stable transduction for lentiviral overexpression**

Normal immortalized MCF-10A cells were used to overexpress MEOX1 with lentiviral vectors. MCF-10A cells were virally transduced with Abmgood pLenti-CMV-RFP-2A-Puro-Blank Control (No. LV591) and Abmgood pLenti-GIII-CMV-hMEOX1-RFP-2A-Puro (No. LV217710) lentiviral vectors. Viral particles were generated at the University of Michigan Vector Core. Cells were infected with lentiviral particles for 24 h using 8 μg/ml of Polybrene. Following 72 h from the start of infection, selection for infected cells was conducted using FACS of RFP-positive MCF-10A pLenti-CMV-RFP-2A-Puro-Blank Control and RFP-positive MCF-10A pLenti-GIII-CMV-hMEOX1-RFP-2A-Puro–transduced cells. MCF-10A cells with pLenti-CMV-RFP-2A-Puro-Blank Control and pLenti-GIII-CMV-hMEOX1-RFP-2A-Puro were hereafter labeled as pLenti control and pLenti MEOX1 overexpression, respectively.

**Two-Step reverse transcription-quantitative PCR (RT-qPCR)**

RNA was isolated from cells using either Life Technologies TRIzol reagent (No. 15596018) or Qiagen RNEasy Kit (No. 74104). RNA isolation was conducted according to the manufacturer’s instructions. Invitrogen SuperScript III First-Strand Synthesis SuperMix (No. 11752-050) was used to make cDNA from isolated RNA. The maximum amount of RNA allotted by the kit was utilized, 1 μg, to make cDNA. Afterward, cDNA was diluted no more than 5-fold to perform qPCR. Applied Biosystems SYBR Green PCR Master Mix (No. 4309155) was used to perform qPCR. All primers were obtained from Integrated DNA Technologies (IDT) and used at a final concentration of 500 nM. The ACTB primer (No. Hs.PT.39a.22214847) was used with forward sequence of 5’-CCTTGCACATGCCGGAG-3’ and reverse sequence of 5’-ACAGAGCCTCGCCTTTG-3’. Although different MEOX1 primers were used to validate results, the main primer used for data obtained in this paper was MEOX1 primer (No. Hs.PT.58.26021003) with forward sequence of 5’-TCATGGAATGTGCCTCC-3’ and reverse sequence of 5’-CAGACTTTCCTGGCGACA-3’.

It is important to note that when performing RT-qPCR for MEOX1, extra care must be taken to handle the RNA samples. MEOX1 RNA expression in breast cancer cell lines is fairly low. MEOX1 was only detected at RNA levels using in vitro breast cancer cell lines; although many attempts were made, no successful antibody was found to detect MEOX1 expression at the protein level.

**Cell proliferation assay**

Following 48 h with transfection of 50 nM Negative Control siRNA and 50 nM MEOX1 siRNA treatments, cells were trypsinized and counted three times. Cells were resuspended to a final concentration of 1 × 10^6 cells/ml and a syringe was utilized with a 23-gauge needle to gently aspirate and dissociate the cells three times to achieve a single cell suspension. Stem Cell Technologies MammaCult Medium (No. 05620) was utilized and supplemented with 4 μg/ml of heparin and 0.48 μg/ml of hydrocortisone. Ultra-Low attachment 6-well–plates were necessary for this experiment to ensure cells would not adhere to the bottom but grow suspended in media. To each well, 3 ml of complete MammaCult medium was added and 5 × 10^3 cells were plated; each treatment group was plated into at least three wells for statistical analysis. Primary mammospheres were allowed to grow undisturbed for 5 days, after which mammospheres >25 μm were counted under a microscope. After counting, mammospheres for each treatment group were collected and combined for secondary mammosphere experiments. Combined wells for each treatment group were trypsinized and counted three times. Cells were resuspended to a final concentration of 1 × 10^6 cells/ml, and again a 23-gauge needle was utilized to gently aspirate and dissociate the cells three times to achieve a single cell suspension. Using Ultra-Low attachment 6-well–plates, 3 ml of complete MammaCult medium was added per well and to each well 5 × 10^3 cells were plated; each treatment group was plated into at least two wells for statistical analysis. All cells from the primary mammospheres were plated for secondary mammosphere formation. Secondary mammospheres were allowed to grow undisturbed for 5 days, after which mammospheres >25 μm were counted under a microscope. Mammosphere formation efficiency (%) was calculated as (number of mammospheres per well/number of cells plated per well) × 100.

**Cell migration and invasion assay**

Following 72 h with transfection of 50 nM Negative Control siRNA and 50 nM MEOX1 siRNA treatments, Corning BioCoat Control Inserts (No. 354578) and Corning BioCoat Matrigel Invasion Chamber (No. 354480) 24-well–plate was utilized for cell migration and invasion assays. Protocol was conducted according to the manufacturer’s instructions with one exception, an optimized seeding density of 7.5 × 10^4 cells per 24-well chamber were plated. For microscopy counting and analysis, cells were fixed and stained using Thermo Scientific Shandon Kwik-Diff Stain Kit (No. 9990701).

**Cloning for short hairpin RNA (shRNA) stable cell lines**

The Addgene Tet-pLKO-puro plasmid (No. 21915) was used to clone doxycycline-inducible MEOX1 shRNA, a gift from Dmitri Wiederschain (72). As such, protocols to clone and establish stable doxycycline-inducible MEOX1 shRNA cell lines were conducted according to Wiederschain and Addgene instruction manuals. Sequences for MEOX1 shRNA were
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obtained from the Mission shRNA Library of The RNAi Consortium; the sequences used for MEOX1 cloning were TRCN0000016108 and TRCN0000016110. TRCN0000016108 contained a top sequence of 5’-CCGGGCTAAGGTTAAGTCGCAATGGGCAGACTTAACCTGG-3’ and a bottom sequence of 5’-AATTAAAAAACAGATGAGACAGGAGGAAATCTCGAGATTTTCTCTCTGTCAATTGG-3’. The Addgene Tet-pLKO-puro-scrambled plasmid (No. 47541) was used as a negative control shRNA, a gift from Charles Rudin (73). The Tet-pLKO-puro-scrambled contained negative control shRNA top sequence of 5’-CCGGGCCTAAGGTTAAGTCGCAATGGGCAGACTTAACCTGG-3’ and a bottom sequence of 5’-AATTAAAAAACAGATGAGACAGGAGGAAATCTCGAGATTTTCTCTCTGTCAATTGG-3’. The following stable cell lines were established using the basal-like MDA-MB-468 TNBC cells: MDA-MB-468 Tet-pLKO-puro-scrambled (labeled as MEOX1 shRNA 1), and MDA-MB-468 Tet-pLKO-puro MEOX1 shRNA TRCN0000016108 (labeled as MEOX1 shRNA 2). Mammary fat pad injections for in vivo tumor growth

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice at 8-10 weeks of age were used for orthotopic fourth inguinal mammary fat pad injections. Xenograft tumor models were established by injecting 5 × 10^6 human breast cancer cells suspended in a 2:3 ratio of 1× PBS/Matrigel, respectively. Three groups of cells were injected, the MDA-MB-468 Tet-pLKO-puro-scrambled (labeled as Scrambled Control shRNA), MDA-MB-468 Tet-pLKO-puro MEOX1 shRNA TRCN0000016108 (labeled as MEOX1 shRNA 1), and MDA-MB-468 Tet-pLKO-puro MEOX1 shRNA TRCN0000016110 (labeled as MEOX1 shRNA 2).

RNA-Seq

BT-549 and MDA-MB-468 cell lines were transfected with 50 nm Negative Control siRNA and 50 nm MEOX1 siRNA mixture. Following 72 h from the start of transfection, cells were harvested by scraping. Harvested cells were pelleted using centrifugation and Pierce RIPA buffer (No. 89900) with Thermo Scientific protease inhibitor mixture (No. 78410), Halt phosphatase inhibitor mixture (No. 78420), and 5 mM EDTA were used to lyse cells for protein extraction. Pierce BCA Assay Kit (No. 23227) was performed to ascertain protein concentrations. A total of 20 μg of protein was used for SDS-PAGE. Nitrocellulose or polyvinylidene difluoride membranes were utilized for protein transfers. Depending on the antibody, blocking was performed for 1 h with either 5% milk or 5% BSA. Primary antibodies were incubated rotating overnight at 4°C. Following several washes, appropriate secondary antibodies conjugated with horseradish peroxidase were added and incubated rotating for 1 h at room temperature. After several washes, enhanced chemiluminescence (ECL) Western blotting substrate was added and blots were imaged using film. JAK/STAT pathway antibodies were purchased from Cell Signaling Technology, which were supplied as STAT Antibody Sampler Kit (No. 9939), Phospho-STAT Antibody Sampler Kit (No. 9914), Phospho-JAK Family Antibody Sampler Kit (No. 97999), and JAK/STAT Pathway Inhibitors Antibody Sampler Kit (No. 8343). Through Santa Cruz Biotechnology, antibodies for STAT5A (No. sc-271542), STAT5B (No. sc-1656), and loading control β-actin (No. sc-
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47778) were purchased. The manufacturer’s instructions were followed for all antibodies to use specific concentrations required for optimal staining.

Statistical analysis

GraphPad Prism 7.0 was used for calculations of all statistical analysis. An unpaired t test was conducted when comparing 2 means of two unmatched groups. One-way ANOVA was conducted when measuring one variable and comparing three or more means. Two-way ANOVA was conducted when determining a change in response based on two factors and comparing three or more means.

Data availability

The RNA-seq data are located in a repository (GSE133927). Other data are to be shared upon request from Duxin Sun (duxins@umich.edu), University of Michigan.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: TNBC, triple-negative breast cancer; MEOX1, molecular target mesenchyme homeobox 1; PTEN, phosphatase and tensin homolog deleted on chromosome 10; qPCR, quantitative PCR; shRNA, short hairpin RNA; DMEM, Dulbecco’s modified Eagle’s medium; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; IPA, integrative pathway analysis; ANOVA, analysis of variance.

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