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Maternal Embryonic Leucine Zipper Kinase (MELK) as a Novel Mediator and Biomarker of Radioresistance in Human Breast Cancer

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Abstract

Purpose: While effective targeted therapies exist for estrogen receptor–positive and HER2-positive breast cancer, no such effective therapies exist for triple-negative breast cancer (TNBC); thus, it is clear that additional targets for radiosensitization and treatment are critically needed.

Experimental Design: Expression microarrays, qRT-PCR, and Western blotting were used to assess MELK RNA and protein expression levels. Clonogenic survival assays were used to quantitate the radiosensitivity of cell lines at baseline and after MELK inhibition. The effect of MELK knockdown on DNA damage repair kinetics was determined using γH2AX staining. The

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Disclosure of Potential Conflicts of Interest

S.G. Zhao is an employee of PFS Genomics and is listed as a coinventor on a patent for compositions and methods for the analysis of radiosensitivity, which is in the process of being licensed to PFS genomics. F.Y. Feng is an employee of PFS Genomics. No potential conflicts of interest were disclosed by the other authors.

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in vivo effect of MELK knockdown on radiosensitivity was performed using mouse xenograft models. Kaplan–Meier analysis was used to estimate local control and survival information, and a Cox proportional hazards model was constructed to identify potential factors impacting local recurrence-free survival.

Results: MELK expression is significantly elevated in breast cancer tissues compared with normal tissue as well as in TNBC compared with non-TNBC. MELK RNA and protein expression is significantly correlated with radioresistance in breast cancer cell lines. Inhibition of MELK (genetically and pharmacologically) induces radiation sensitivity *in vitro* and significantly delayed tumor growth *in vivo* in multiple models. Kaplan–Meier survival and multivariable analyses identify increasing MELK expression as being the strongest predictor of radioresistance and increased local recurrence in multiple independent datasets.

Conclusions: Here, we identify MELK as a potential biomarker of radioresistance and target for radiosensitization in TNBC. Our results support the rationale for developing clinical strategies to inhibit MELK as a novel target in TNBC.

Introduction

Recently, locoregional control of breast cancer has been shown to improve both distant disease-free and overall survival in patients with newly diagnosed, early-stage disease (1, 2). Therefore, optimal local control by surgical and radiation therapies is important for such patients. Other than size, lymph node, and margin status, few if any markers provide an indication of either risk of subsequent LR recurrence either in the absence, or presence, of radiation. Prognostic markers might provide an indication of which patients might avoid costly and toxic treatments, and predictive markers of radiation resistance might provide insight into novel radiation-sensitizing strategies to reduce the odds of locoregional recurrence, and subsequently, distant recurrence and mortality.

In this regard, locoregional recurrence is higher in patients with estrogen receptor (ER)-, progesterone receptor (PgR)-, and HER2-negative breast cancers. These so-called “triple-negative” breast cancers (TNBC) are not only more likely to recur in the absence of radiation after either mastectomy or breast-conserving surgery (BCS) but also appear to have relative radioresistance (3–5). However, both the prognostic and predictive role for locoregional recurrence and radiation resistance of these three markers is relative and not very helpful in guiding patient treatment (4, 6–8). Furthermore, there has been a relative absence of clinically effective radiation sensitizers in women with treatment-refractory breast cancers or for women who are at high risk of locoregional recurrence. Given the lack of targeted agents for triple-negative disease and their relative radiation insensitivity as evidenced by their increased locoregional recurrence risk, it is clear that additional targets for radiosensitization are critically needed, including those that are selective for TNBC.

We have previously identified one such potential target, maternal embryonic leucine zipper kinase (MELK; ref. 9). MELK is an atypical member of the snf1/AMPK family of serine/threonine kinases that has also been shown to be enriched in TNBC (10, 11). This family is largely associated with cell survival under conditions of environmental challenge, such as nutrient starvation (12, 13). Previous studies, however, have demonstrated that MELK

may regulate other important processes, including stem cell self-renewal through control of the cell cycle (14). Likewise, MELK has been identified as a cell-cycle modulator in tumor cell lines and was recently identified as an important target for certain solid malignancies, including brain, breast, colorectal, lung, and ovarian cancers (11, 15, 16). MELK has been identified as an inhibitor of apoptosis by interacting with Bcl-Gl and may play a role in mammary tumor initiation (17–19). The role of MELK as a mediator of radiation resistance in TNBC, however, remains unexplored.

In this study, we identify MELK as a novel therapeutic target in triple-negative and treatment-refractory breast cancer. We show that MELK expression is associated with resistance to radiation treatment both *in vitro* and *in vivo*. MELK expression is limited primarily to cancerous tissue, primarily breast cancer, and is not expressed in normal tissues, suggesting a potentially favorable therapeutic index. Mechanistic studies demonstrate that MELK expression is associated with repair of double-stranded DNA (dsDNA) breaks induced by ionizing. Finally, our data suggest the prognostic and radiation resistance prediction role of MELK expression in human breast tumors. Taken together, these results suggest that MELK may be a clinically relevant biomarker and potential therapeutic target in TNBC and radiation treatment-refractory breast cancer.

Materials and Methods

Please refer to the detailed materials, methods, and statistical descriptions included in the Supplementary Material for full details. A brief description is included here.

Cell culture and cell lines

Breast cancer cells were propagated from frozen samples in cell culture media and passaged when reaching confluence. Cell lines were chosen to include an appropriate representation of all molecular subtypes. All cell lines were purchased between July 2012 and August 2015 from ATCC (except the ACC cell lines) and the remainder (all ACC cell lines) from the Deutsche Sammlung von Mikroorganismens und Zellkulturen GmbH (DSMZ). All cell lines were authenticated and genotyped immediately prior to evaluation at the University of Michigan (Ann Arbor, MI) DNA Sequencing core facility by fragment analysis and ProfilerID utilizing the AmpFLSTR Identifier Plus PCR Kit (Life Technologies, cat # 4322288) run on an Applied Biosystems AB 3730XL 96-capillary DNA analyzer.

RNA isolation and quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) and an RNeasy kit (Qiagen) according to manufacturers' instructions. Total RNA was reverse transcribed into cDNA using Super-Script III and random primers (Invitrogen). Quantitative PCR (qPCR) was performed using SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems 7900HT Real-Time System.

Western blot analysis

For protein isolation from tissue culture cell lines, cells were washed once with ice-cold PBS and lysed in protein lysis buffer. Western blot analysis was performed as described

previously (20). A detailed description of methods and antibodies is included in the Supplementary Materials and Methods.

siRNA and short hairpin RNA experiments

siRNA experiments utilized ON-TARGET plus SMARTpool siRNA (Thermo Scientific) targeting MELK or nontargeting control (nontargeting pool, catalog no. D-001810–10-50, Invitrogen) according to the manufacturer's instructions. The pTRIPZ lentiviral system with MELK-inducible shRNA transfection starter kit was purchased from Thermo Scientific using cat #RHS4696–200703132 and cat #RHS4696–200691582 for non-template control and shMELK. Stable cell lines were generated using lentiviral transduction. Clones were selected and screened for both RFP and MELK expression changes and were used as pools and as selected stable clones in all *in vitro* and *in vivo* experiments.

Clonogenic survival assays

Exponentially growing cells were treated with MELK knockdown and/or radiation at doses as indicated with plating efficiency correction for all experiments. Drug cytotoxicity was calculated as the ratio of surviving drug-treated cells relative to untreated control cells. Cell survival curves were fitted using the linear–quadratic equation with radiation enhancement ratio (EnhR) calculated as the ratio of the mean inactivation dose under control conditions divided by the mean inactivation dose under gene knockdown conditions.

Irradiation

Irradiation was carried out using a Philips RT250 (Kimtron Medical) at a dose rate of about 2 Gy/minute in the University of Michigan Comprehensive Cancer Center Experimental Irradiation Core.

Proliferation assays

Cells were plated in 48-well plates at various concentrations (15,000 cells per well for BT-549 and MCF-7; 10,000 cells per well for MDA-MB-231) and treated with the indicated conditions and placed in the Incucyte System (Incucyte ZOOM, Essen BioScience). Cell growth measurements were taken every 2 hours.

Flow cytometric apoptosis assays

Cells were transfected or treated with inhibitor as indicated. Forty-eight hours after transfection, the cells were harvested, and the apoptosis assay utilizing cleaved PARP was performed as described above. Apoptotic assays by flow cytometry were performed using ApoScreen Annexin V Apoptosis Kit (Southern Biotech #10010–02), as per the manufacturer's protocol.

Mouse xenograft experiments

After tumors reached 50 to 100 mm^3, shMELK expression was induced by doxycycline in the experimental arm with the control mice receiving no doxycycline. Each group contained 16 to 20 xenografts in each treatment arm. Growth in tumor volume was recorded three times per week after shaving of the bilateral flanks by using digital calipers and tumor

volumes were calculated. The fractional product method was used to determine additive versus synergistic effects as described previously (21). All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan and conform to their relevant regulatory standards.

γH2AX foci formation

Analysis of γH2AX by flow cytometry was performed as described previously (22). Cells with ≥10 γH2AX foci were scored as positive and compared for statistical analyses.

Patient cohorts

A publicly available clinical cohort with gene expression and locoregional information was utilized for biomarker assessment (Servant). It included 343 patients with early-stage breast cancer treated with BCS and postoperative radiotherapy (23). Gene expression from an additional dataset (Wang) consisting of patients with lymph node–negative breast cancer who were treated with BCS (219 patients) or mastectomy (67 patients) from 1980 to 1995 (24). These patients also received radiotherapy when indicated (87%). Fewer than 40% of the patients in either dataset received any adjuvant systemic therapy. Local recurrence-free survival was tracked in all patients. All patients from both datasets were used in the analysis, and complete patient and cohort characteristics are included in the Supplementary Tables S2 and S3 for each dataset. Specimen characteristics and handling were described previously (23, 24). Data are presented in accordance with the REMARK guidelines, and no patients from these studies were excluded from these analyses. Please refer to the original cited publications for full details of the Institutional Review Board (IRB) approval.

Microarrays

Normalized expression data for the cell lines were downloaded from the EMBL-EBI ArrayExpress website as described in the original publication (24, 25).

Results

MELK is more highly expressed in breast tumors compared with normal breast tissue and is enriched in basal-like and triple-negative breast cancers

Our previous work using gene expression profiling to identify differentially expressed kinases between ER-positive and ER-negative tumors identified MELK as one of the most differentially expressed kinases in ER-negative breast cancer (9). In this study, we sought to further examine the association between MELK expression and the intrinsic subtypes of breast cancer by interrogating the Cancer Genome Atlas (TCGA) breast dataset (26–28). MELK RNA expression is significantly increased in human breast tumors compared with normal breast tissue with little to no expression identified in normal tissues (Fig. 1A). In addition, serial analysis of gene expression (SAGE) analysis demonstrated markedly elevated levels of MELK RNA expression in breast cancers (as well as certain genitourinary malignancies) and absent to near-absent expression in normal tissues suggesting a potentially favorable therapeutic index (Supplementary Fig. S1) when treating with MELK inhibitors. While MELK expression was nearly absent in normal breast tissues, MELK expression was heterogeneous across breast tumors with approximately 20%

of human tumors demonstrating markedly elevated levels of expression. Further analysis of the TCGA dataset demonstrates that MELK expression is significantly elevated in the basal-like, HER2-amplified, and luminal B subtypes when compared with either the luminal A or normal-like subtypes (Fig. 1B). Furthermore, MELK RNA expression was highest in the triple-negative subtype of breast tumors (Fig. 1C). We validated the increased expression in tumor specimens compared with normal breast tissue (from an institutionally assembled dataset of breast reduction mammoplasties and breast tumors) and in ER-negative breast tumors compared with ER-positive tumors (Fig. 1D and E).

MELK is more highly expressed in ER-negative breast cancer cell lines

The association of MELK expression and basal-like breast cancer was further supported in data derived from 51 *in vitro* cultured human breast cancer cell lines (25). Using this data, MELK expression was significantly higher in the largely ER-negative (basal A and basal B) breast cancer cell lines compared with the ER-positive luminal breast cancer cell lines (Supplementary Fig. S2A and S2B). As this gene expression data indicated that MELK was more highly expressed in ER-negative breast cancer cell lines, we chose 12 ER-positive or ER-negative breast cancer cell lines and measured the expression of MELK RNA under basal growth conditions using qRT-PCR. MELK expression (RNA) was again found to be significantly elevated in the ER-negative breast cancer cell lines ($P = 0.007$) as compared with the ER-positive breast cancer cell lines (Supplementary Fig. S2C).

We next verified that the protein levels of MELK were also increased in ER-negative breast cancer cell lines. Using Western blot analysis, we demonstrated that MELK was more highly expressed in ER-negative breast cancer cell lines compared with ER-positive breast cancer cell lines ($P = 0.02$; Supplementary Fig. S3A–S3C). Furthermore, total MELK protein and RNA expression levels were significantly correlated across all breast cancer cell lines with a correlation coefficient of 0.88 ($P < 0.01$; Supplementary Fig. S3D). This RNA and protein expression data was used to identify cell lines for future experimentation in these studies.

MELK expression is correlated with radioresistance *in vitro*

Recognizing that TNBCs demonstrate increased levels of radiation resistance clinically, we sought to determine what, if any, role MELK played in the radioresistance phenotype in human tumors. We began by performing clonogenic survival assays on 21 breast cancer cells lines chosen to represent the heterogeneity common in human breast cancer. Doses of radiation between 1 and 6 Gy were utilized, and the area under the clonogenic survival curve (AUC) was calculated for each of the 21 breast cancer cell lines. Higher AUC values are associated with increasing radiation resistance as higher doses of radiation are necessary to elicit and equieffective cell killing. In addition, MELK expression was assessed in the same 21 breast cancer cell lines, and correlation coefficients were calculated between MELK expression levels and radiation sensitivity as assessed by clonogenic survival assays (AUC value). This analysis demonstrated a significant correlation between MELK RNA expression, and the intrinsic radiosensitivity of the breast cancer cell lines with increasing MELK RNA expression in the breast cancer cell lines significantly correlated with increasing radioresistance ($R^2 = 0.62$, $P < 0.005$; Fig. 2A). Similarly, MELK protein

expression was even more significantly correlated with increasing radioresistance (R^2 = 0.88, $P < 0.001$; Fig. 2B).

MELK knockdown confers radiosensitivity in TNBC cells

We then more fully explored the role of MELK in regulating radioresistance in ER-negative breast cancer using two independent ER-negative breast cancer cell lines found to have high MELK expression. Clonogenic survival assays were performed on cell lines with high MELK expression (MDA-MD-231 and BT-549) using scrambled control siRNA oligonucleotides or two independent siRNA oligonucleotides designed to inhibit MELK expression. Clonogenic survival curves show potent and consistent radiosensitization with MELK knockdown using these two independent MELK siRNA oligonucleotides in MDA-MB-231 cells with enhancement ratios of 1.55 to 1.62 using MELK knockdown alone (Fig. 2C). For comparison, the well-characterized radiosensitizing drug cisplatin demonstrates enhancement ratios of 1.2 to 1.3 in cancer cell lines (29, 30). There was also a significant difference in the surviving fraction after 2 Gy (the dose of daily radiation used clinically for patients treated for breast cancer) in these cells (Supplementary Fig. S4A). Confirmation in the independent TNBC cell line BT-549 again showed significant radiosensitization (enhancement ratios ranged from 1.48–1.52) with MELK knockdown using the same control and MELK siRNA oligonucleotides (Fig. 2D). MELK knockdown was confirmed at both the RNA and protein levels (protein levels shown in Fig. 2E and F) with limited toxicity with MELK knockdown alone in both cell lines with a significant difference in the surviving fraction after 2 Gy values (Supplementary Fig. S4A–S4C). This radiation sensitization was also confirmed using two additional siRNA constructs designed against different exons of MELK to confirm specificity (Supplementary Fig. S4D and S4E). To confirm that this effect was not transient and in preparation for future *in vivo* xenograft experiments, shRNA constructs were generated and cell lines were transduced to make stable, doxycycline-inducible shMELK cell lines. Utilizing these transduced, stable MDA-MB-231 cell lines with inducible shMELK, clonogenic survival assays were again performed after induction of MELK knockdown by doxycycline induction. As in the siMELK experiments, MELK knockdown showed significant radiosensitization utilizing two independent shMELK constructs with radiation enhancement ratios ranging from 1.44 and 1.52. There was no effect on radiosensitization with doxycycline alone or induction of a control nontargeted shRNA construct (shNT; Supplementary Fig. S5A).

To further investigate whether MELK kinase function, not just protein level, was necessary for this radiosensitization phenotype, the recently published MELK inhibitor OTSSP167 was used in clonogenic survival assays (31). As with genetic manipulation of MELK expression using siMELK and shMELK constructs, pharmacologic inhibition of MELK kinase function significantly radiosensitized MDA-MB-231 breast cancer cell lines with radiation enhancement ratios of 1.61 and 1.68 at OTSSP167 concentrations of 100 nmol/L and 1 μmol/L, respectively (Supplementary Fig. S5B). Similar findings were demonstrated using the MELK inhibitor in an independent triple-negative cell line BT549 (data not shown). Thus, not only was MELK expression necessary for radioresistance but also its kinase function was needed to confer resistance to ionizing radiation.

MELK overexpression confers radioresistance in ER-positive breast cancer cells with low baseline MELK expression

To demonstrate causality, we next explored whether overexpression of MELK protein in a breast cancer cell line with low MELK expression, in this case the ER-positive breast cancer cell lines MCF-7, would confer radioresistance. As compared with the TNBC cell lines MDA-MB-231 and BT-549, MCF-7 cells are significantly more sensitive to the effects of ionizing radiation at baseline and are considered a radiosensitive cell line. Overexpression of MELK protein conferred radioresistance in MCF-7 cells (Fig. 2G) with a subsequent significant increase in the SF 2 Gy values. MELK protein and RNA overexpression was confirmed using Western blot and qRT-PCR analyses (Fig. 2H and I). Thus, knockdown of MELK protein expression and inhibition of MELK kinase function using the siMELK and shMELK constructs and the MELK inhibitor OTSSP167 *in vitro* was sufficient to confer significant radiosensitization in two independent radioresistant cell lines with high MELK expression. Similarly, MELK overexpression in a radiosensitive cell line with low MELK expression was sufficient to confer radioresistance. To confirm that the effects were consistent with radiosensitization by MELK inhibition and were not solely a function of decreased proliferation or increased apoptosis, we assessed the effects of MELK inhibition (genetic or pharmacologic) or overexpression on the growth and apoptotic rates of breast cancer cell lines (Supplementary Figs. S6–S8). These data indicate that MELK knockdown or inhibition had at best a modest effect on proliferation and little effect on apoptosis rates in most cell lines examined.

GSEA analysis identifies DNA damage repair strongly as being strongly associated with MELK expression

To gain insight into the potential mechanisms whereby MELK was contributing to radioresistance, we performed gene set enrichment analysis (GSEA) to identify concepts associated with MELK expression across all published gene expression datasets. MELK gene expression was correlated to every sequenced gene in the TCGA breast dataset and genes that were significantly positively or negatively correlated with MELK expression were retained within the gene set. These gene lists were then input into GSEA as described previously (32). GSEA identified that of the top 10 nominated positively associated concepts, three were DNA damage–related, and four were cell-cycle regulation–related (Supplementary Fig. S9A, negatively associated concepts in Supplementary Fig. S9B). These include concepts related to response to radiation-induced DNA damage at 6 and 24 hours (Supplementary Fig. S9C and S9D). In addition, molecular concept mapping was performed and demonstrates DNA damage repair and cell-cycle regulation as the two most strongly nominated biologic concepts (Supplementary Fig. S10A). Furthermore, Ingenuity Pathway analysis (IPA) identified the role of BRCA1 in DNA damage response and the G_2–M DNA damage repair checkpoint as the most significantly correlated canonical pathways (Supplementary Fig. S10B), suggesting the critical role of MELK in repair of ionizing radiation–induced DNA damage. In addition, many of the genes associated with DNA repair mediated by homologous recombination were significantly and positively correlated MELK expression with the BRCA1-mediated DNA repair pathway genes, including FANC family proteins, CHK1, CHK2, PLK1, and complex B proteins most significantly involved (Supplementary Fig. S10B).

dsDNA repair is inhibited by MELK knockdown or inhibition

Having identified DNA damage repair as the most significant concept in GSEA analysis, we interrogated the role of MELK in DNA damage repair. As ionizing radiation confers lethality through the introduction of dsDNA breaks, we sought to assess what, if any, role MELK played in dsDNA damage repair using γH2AX foci formation assays. γH2AX assays were performed and quantitated using two independent methods that included manual counting of γH2AX foci (Fig. 3A–C) or using flow cytometry and sorting for phospho-H2AX–positive cells (Fig. 3D and E). MELK knockdown itself did not significantly impact dsDNA break formation (Fig. 3B and D). As expected, dsDNA damage was significantly increased with radiation treatment alone (2 Gy) and this damage persisted significantly longer (4 and 16 hours) in the cells with MELK knockdown (using siRNA) compared with control transfected cells (Fig. 3B and D). Thus, MELK expression significantly impacted the degree and rate of dsDNA break repair. Similarly, when we used the previously developed targeted MELK inhibitor OTSSP167, dsDNA damage persisted significantly longer and to a greater extent in the MELK inhibitor–treated cells compared with control vehicle–treated cells at 4, 16, and 24 hours (Fig. 3C and E), suggesting the kinase function of MELK was critical for repair and resolution of dsDNA breaks.

MELK knockdown or inhibition significantly delays xenograft tumor growth in combination with radiation therapy

Having demonstrated that MELK inhibition results in radiosensitization of multiple breast cancer cell lines, we sought to validate these findings in a mouse xenograft model. Using inducible shMELK constructs under doxycycline control, SCID mice were injected with transduced MDA-MB-231 cells. After tumors reached sufficient size, shMELK expression was induced by doxycycline in the experimental arm. There were four treatment groups that included a control group, MELK knockdown alone, radiation therapy alone, and combination treatment (see Supplementary Methods for full details). Knockdown of MELK alone or radiation alone resulted in decreased tumor volume growth in the xenograft model (Fig. 4A), but the combination of radiation and MELK knockdown resulted in a statistically significant ($P < 0.0001$) reduction in tumor growth compared with radiation alone, and a tumor volume doubling time nearly four times as long as treatment with radiation alone (Fig. 4B). Furthermore, analysis of the enhanced combination effect at different time points demonstrated considerable synergism between the radiation and MELK knockdown treatments (Supplementary Table S1). The indices (R) for combination therapy with MELK inhibition and radiation were >1, indicating a synergistic interaction between the drugs. To confirm that MELK kinase function, and not merely expression level, was necessary for radiosensitization *in vivo*, similar experiments were performed using wild-type MDA-MB-231 cells injected into the bilateral flanks of SCID mice. These mice were treated with the MELK inhibitor OTSSP167 daily via oral gavage after tumors reached sufficient size with inhibitor treatment initiated 24 hours before radiation administration. As with the MELK knockdown experiment, MELK inhibition with the oral inhibitor OTSSP167 led to a similar synergism with radiation treatment and marked radiosensitization (Fig. 4C and Supplementary Table S1) and delay of tumor doubling time (Fig. 4D). MELK expression was assessed by qRT-PCR from xenograft tumors harvested during the fourth week of the experiment to confirm effective targeting in the shMELK group (Fig. 4E). In addition,

treatment with the MELK inhibitor OTSSP167 did not affect MELK expression levels in the harvested xenograft tumors (Fig. 4E) suggesting that the radiosensitivity conferred by the MELK inhibitor was related to inhibition of MELK kinase function. In addition, treatment with MELK inhibitor did not result in significant toxicity in the mice, a toxicity profile consistent with reports from other groups (31). An outline of the experimental design is depicted in Fig. 4F.

MELK expression is prognostic in breast cancer and predictive of local recurrence

While our studies identified MELK as being implicated in radioresistance *in vitro* and *in vivo*, we wanted to determine whether MELK expression was a predictor of response to ionizing radiation and associated with poorer prognosis in breast cancer patients treated with ionizing radiation. For these studies, we analyzed the local recurrence-free survival data from several different, publically available datasets, with patient and dataset characteristics listed in Supplementary Tables S2 and S3. All datasets had a minimum of 12-year follow-up and local recurrences had been tracked in these cohorts. In addition, MELK expression levels were available for all tumor samples. The Wang dataset included patients with lymph node–negative breast cancer who were treated with BCS (219 patients) or modified radical mastectomies (MRM; 67 patients) from 1980 to 95. These patients also received radiotherapy when indicated (87%), but most did not receive systemic chemotherapy. In this dataset, we first divided tumors by median level of MELK expression. Kaplan–Meier analysis of the local recurrence-free survival between the different groups (higher than median vs. lower than median expression) showed that women who had higher MELK expression had a significantly increased risk of local recurrence, even after radiation treatment (Fig. 5A). In addition, when expression was ordered in descending order and patients were divided into quartiles on the basis of the level of MELK expression, a step-like increase in local recurrence was noted as the level of MELK expression increased (Fig. 5B). Similarly in the Servant dataset, which consisted of 343 early-stage node-negative patients managed with BCS and treated with radiation the same pattern of increased local recurrence after ionizing radiation was found among those patients whose tumors had higher than median expression of MELK (Fig. 5C). Again, when the cohort was divided into quartiles, the increasing rates of local recurrence were noted as the levels of MELK expression increased (Fig. 5D). Thus, in keeping with our preliminary *in vitro* and *in vivo* data, MELK expression levels were significantly and repeatedly associated with increased rates of local recurrence after ionizing radiation.

Although Kaplan–Meier analysis in multiple datasets suggested that MELK expression may in itself be prognostic of overall survival and predictive of response to ionizing radiation, we performed uni- and multivariate Cox proportional hazards analysis using local recurrence as an endpoint to determine whether MELK expression was independently prognostic, including all available clinical and biologic characteristics of the tumor that may affect local control in the model. This analysis was performed on the Servant dataset, as it had the most complete and updated clinical, pathologic, and local recurrence-specific information. As expected, univariate analysis identified multiple factors significantly associated with local recurrence risk, but in multivariable analysis, MELK expression level, analyzed as a continuous variable, outperformed all other prognostic clinical or pathologic variables,

including the intrinsic breast cancer subtype (Table 1). Significance increased, as does the HR, when analyzed as an ordinal variable.

Discussion

In this report, we identify maternal embryonic leucine zipper kinase (MELK) as one of the most highly differentially expressed kinases in ER-negative breast cancers as compared with ER-positive breast cancers. In addition, MELK is overexpressed in TNBC and tumors that give rise to local recurrences, including a disproportionately high number of radiation-refractory tumors. Further analysis revealed that MELK is not normally expressed at appreciable levels in most normal tissues, including normal breast tissue, but is more highly expressed in breast tumors, especially ER-negative tumors, demonstrating a potentially favorable therapeutic index when translated clinically. MELK expression is strongly correlated with sensitivity to radiation, and inhibition of MELK expression or function leads to significant radiosensitization *in vitro* and *in vivo* through impaired DNA damage repair. Finally, increasing MELK expression is significantly associated with increasing rates of local failure after radiotherapy across multiple datasets and multivariable analysis identifies MELK expression as the strongest factor associated with poor local control. These results suggest that women whose tumors have high MELK expression have a poor prognosis and derive less benefit from radiotherapy and therefore may benefit from more aggressive treatment. In addition, this study identifies MELK itself as a potential target for the treatment in TNBC.

TNBC has consistently been shown to portend a poorer response to radiotherapy, increased rates of local recurrence, and an overall poorer prognosis (6, 33). Recent efforts have sought to identify actionable targets in TNBC, including those that may radiosensitize these tumors. A number of inhibitors of peptide growth factor pathways, such as EGF receptor (EGFR), the insulin-like growth factor receptor (IGFR), FGF receptors (FGFR), VEGF pathways, have been studied in numerous clinical trials with limited success (34–36). Additional targeted therapies, including inhibitors of PI3K, PARP-1, CHK1, AR, and Src, are in various stages of clinical trials in breast cancer. Although these therapies may prove effective in treating subsets of women with breast cancer, it is clear that additional therapies are critically needed. Many of these therapies have side effects that limit their clinical utility, and the problem of drug resistance remains a substantial limitation to their use. In addition, although these targets hold promise for the treatment of tumors that express the aforementioned markers, many ER-negative tumors do not express any of these targets. These studies credential MELK as a possible additional target for the more effective treatment, with an expression pattern that suggests a favorable therapeutic index.

While these results show radiosensitization of TN and basal-like breast cancers with the inhibition of MELK both *in vitro* and *in vivo*, the mechanism of radiosensitization remains to be fully elucidated. Previous groups have demonstrated that in gliomas, MELK knockdown leads to cellular senescence, cell-cycle arrest, and increased replicative stress secondary to the increase in dsDNA breaks (37). This is mediated, in part, by p21 whose expression is increased by MELK depletion. This, in turn, activates ATM, Chk2, and p53 sequentially causing cell-cycle arrest and accumulation of DNA damage at stalled

replication forks. This same group subsequently demonstrated a similar mechanism when treating with a novel MELK inhibitor (upregulation of p21 leading to activation of ATM, Chk2, and p53) allowing the cancerous cells to continue proliferation in the presence of replicative stress (37, 38). It is unclear what, if any, role this mechanism plays in radioresistance of triple-negative and basal-like breast cancers as the vast majority of these tumors, and all of the cell lines used in this study, harbor p53 mutations. More interestingly, however, is the observation that MELK knockdown or inhibition leads to cell-cycle arrest in early S-phase or in G_2–M (39). As these phases correspond to time when cells are most sensitive to the effects of ionizing radiation, this may be a mechanism whereby MELK inhibition leads to radiosensitization. Furthermore, it remains to be seen what affect MELK function has on either nonhomologous end joining or homologous recombination to impact DNA damage repair. A more complete understanding of the mechanism by which MELK contributes to the radioresistance phenotype will allow for the rationale design of strategies to interfere with this resistance and is the subject of ongoing investigation.

Given the lack of targeted agents for triple-negative disease and their relative radiation insensitivity, as evidenced by their increased locoregional recurrence risk, it is clear that additional targets for radiosensitization are critically needed, including those that are selective for TNBC. This report identifies MELK as one such targetable kinase. MELK represents an ideal molecular target, as it demonstrates many of the characteristics necessary for effective targeted therapies. MELK protein is expressed in cancerous tissue but not normal tissue suggesting an ideal candidate with a broad therapeutic window. Its kinase function, which in this report was shown to be necessary for the effective repair of radiation-induced DNA damage, is imminently targetable as the crystal structure of the MELK has already been established (40, 41). Furthermore, MELK inhibitors have already been developed and have shown efficacy in *in vitro* and *in vivo* model systems, and additional inhibitors are currently in various stages of development (31). In addition, MELK expression is high enough in breast cancers, especially TNBC, to be clinically relevant, and as demonstrated in this report, it serves as both a prognostic and a predictive biomarker in predicting response to radiation treatment. Finally, MELK functionally plays a role in several of the processes that are “hallmarks” of cancer including proliferation, migration, invasion, cell-cycle regulation, and DNA damage repair. Thus, while there has been a relative absence of clinically available radiation sensitizers in women with treatment refractory or TNBC, MELK represents a novel therapeutic target that holds promise for the more effective treatment of this deadly disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Sustained locoregional and distant control of breast cancer is a significant issue in patients with breast cancer, especially in women who present with triple-negative breast tumors. Given the unsatisfactory outcomes with standard treatment approaches, there is a clear need for intensification of treatment, including radiotherapy for these patients. This study identifies MELK as being significantly overexpressed in triple-negative and basal-like tumors. It also demonstrates that MELK expression is associated with radiation resistance and is associated with poorer local control both *in vitro* and *in vivo*. Furthermore, survival analysis of patients with breast cancer shows that those patients whose tumors have high expression of MELK have a significantly poorer prognosis than patients with low expression of MELK, as well as an increased risk of local recurrence after radiation alone. Thus, inhibition of MELK represents a novel and promising strategy for radiosensitizing aggressive tumors.

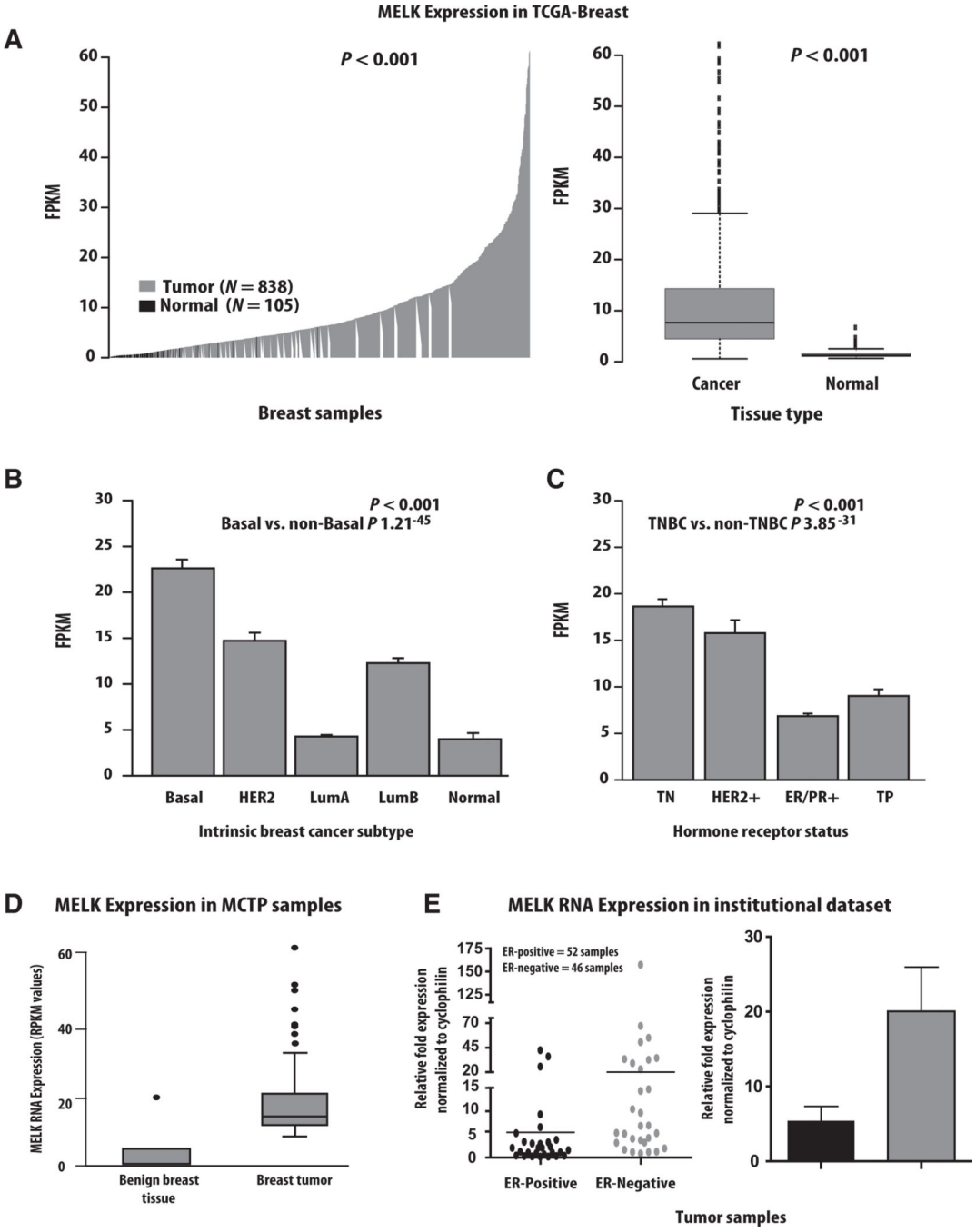

Figure 1.

MELK is more highly expressed in cancerous tissue and TNBC. Analysis of TCGA breast dataset demonstrates MELK expression is significantly higher in breast tumors (in red) than in normal breast tissue (in green) with FPKM values on the *y*-axis and individual tumor samples from cancer versus normal on the *x*-axis (**A**). Error bars represent ±SD. MELK expression is also significantly elevated in basal-like and TNBCs in the TCGA dataset (**B** and **C**). Error bars represent ±SEM. The expression of MELK in 180 breast normal and tumor samples (22 reduction mammoplasty normal and 158 tumors) was measured

using RNA-sequencing analysis from the University of Michigan Translational Pathology databank (**D**). Data are depicted as absolute RPKM values. Differential expression between ER-negative and ER-positive human breast tumors was also confirmed in an institutional breast tumor database using qRT-PCR analysis (**E**). Expression is depicted normalized to control with error bars representing ±SEM.

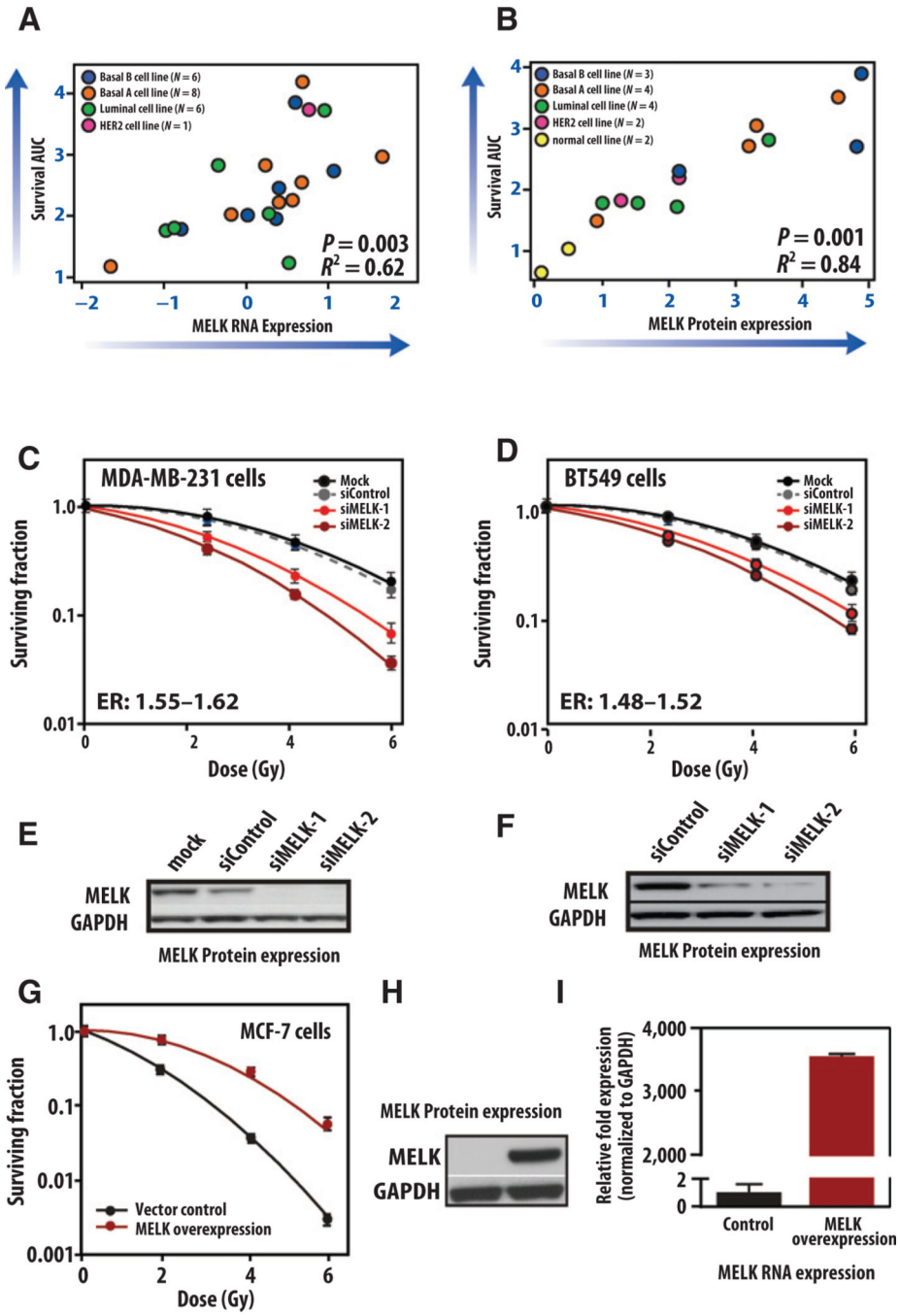

Figure 2.

MELK expression is associated with radioresistance. Intrinsic radiosensitivity of 21 breast cancer cell lines (as measured by clonogenic survival assay area under the survival curve, AUC) was assessed and correlated to MELK RNA expression using Pearson correlation. Each dot represents an individual cell line with colors corresponding to intrinsic subtype. Mean centered $\log_2$ RNA expression is depicted on the *x*-axis and the survival AUC from clonogenic survival assays are depicted on the *y*-axis (**A**). Intrinsic radiosensitivity was also correlated to MELK protein expression in 15 breast cancer cell lines with MELK

expression relative to the expression in MCF-7 cells as determined by Western blotting (**B**). Using siRNA knockdown of MELK expression in a radiation-resistant breast cancer cell lines with high baseline MELK expression (MDA-MB-231 and BT549), radiation sensitivity was assessed using clonogenic survival assays. Knockdown of MELK expression confers radiation sensitivity with limited toxicity with an enhancement ratio of 1.55 to 1.62 in MDA-MD-231 cells (**C**) and 1.48 to 1.52 in BT549 cells (**D**). MELK knockdown was confirmed in both experiments using Western blotting for MELK expression (**E** and **F**). MELK overexpression in the radiosensitive ER-positive breast cancer cell line MCF-7, with low baseline MELK expression and high radiosensitivity confers radioresistance (**G**). MELK overexpression was confirmed at the protein and RNA level (**H** and **I**). All experiments were repeated in triplicate with error bars ±SEM.

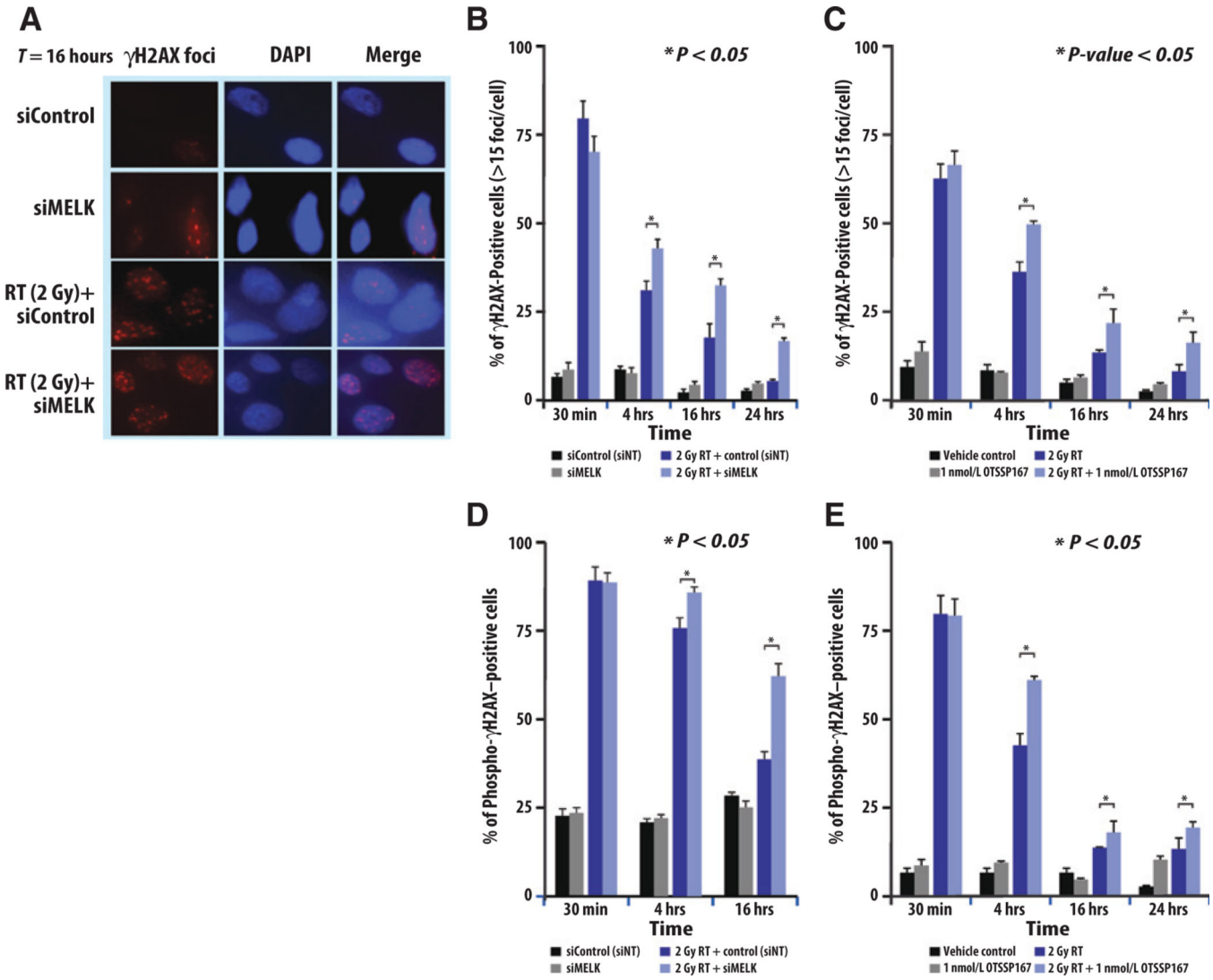

Figure 3.

MELK knockdown significantly delays repair of dsDNA breaks after ionizing radiation. Representative images of γH2AX foci at 16 hours are depicted in **A.** Using siRNA directed against MELK, the effect of MELK knockdown on γH2AX foci formation (**B**) or fluorescence staining by flow cytometry (**D**) was evaluated at various times (30 minutes, 4 hours, 16 hours, 24 hours) after 2 Gy of ionizing radiation. The effect on dsDNA break repair caused by inhibition of MELK kinase function using the MELK inhibitor OTSSP167 was also assessed in time course by foci formation (**C**) or flow cytometry (**E**). Each experiment was run in triplicate three independent times. Similar results were found using the cell line BT549 (data not shown). Error bars represent SD.

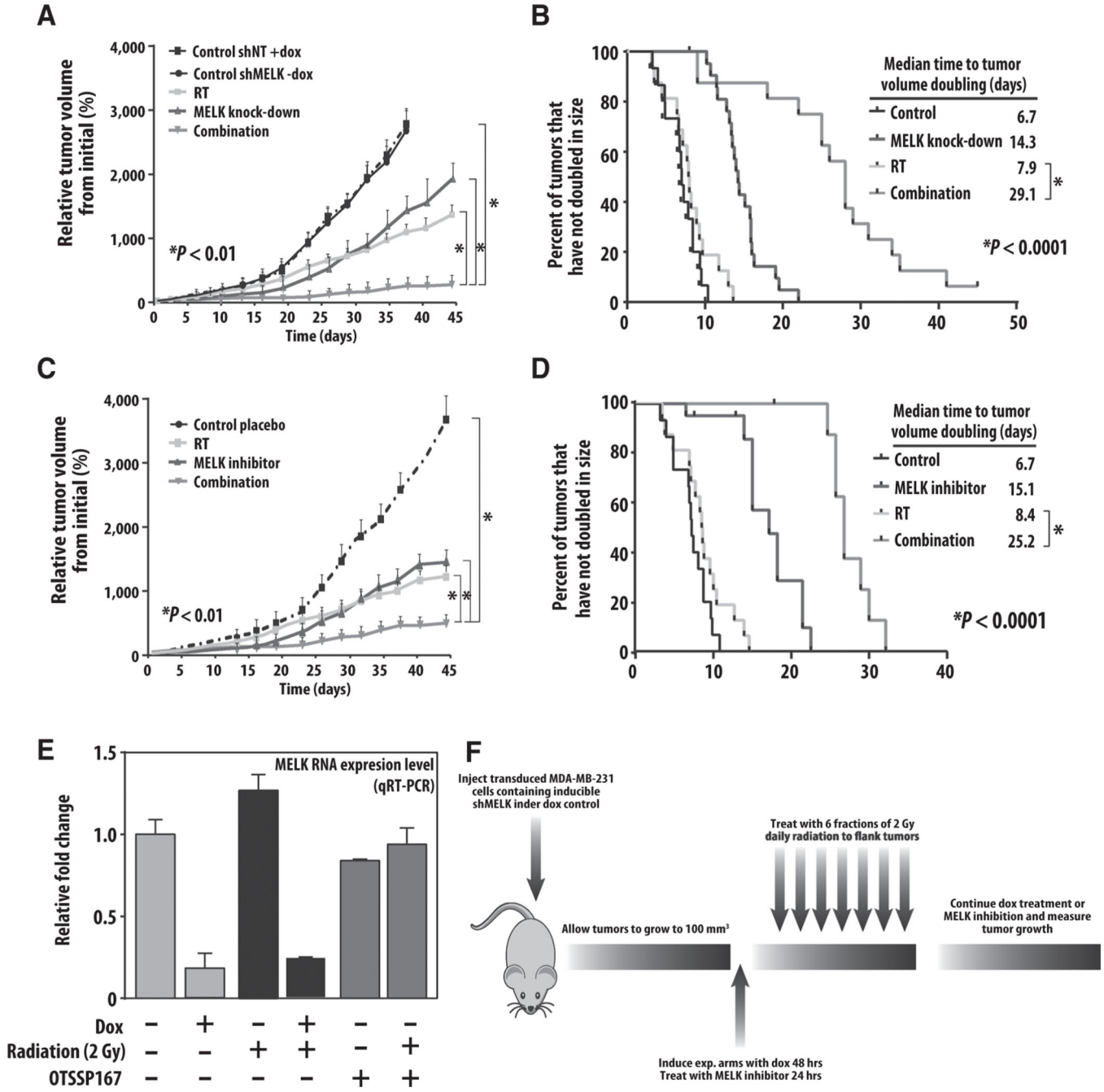

Figure 4.

MELK inhibition significantly reduces xenograft tumor doubling time compared with radiation alone. Knockdown of MELK alone or radiation alone resulted in decreased tumor volume growth in the xenograft model (**A**), but the combination of radiation and MELK knockdown resulted in a synergistic and statistically significant ($P < 0.01$) reduction in tumor growth compared with radiation alone, and a tumor volume doubling time nearly four times as long as treatment with radiation alone (**B**). A similar experiment was performed using wild-type MDA-MB-231 cells injected as above, but this time treatment was with the MELK inhibitor OTSSP167 treated at 10 mg/kg daily by oral gavage. While radiation

and MELK inhibitor alone did delay tumor doubling time slightly, combination therapy was significantly more effective at delay tumor growth and doubling time (**C** and **D**). MELK expression was assessed by qRT-PCR from xenograft tumors harvested during the fourth week of the experiment (**E**). A depiction of the experimental design is shown (**F**). Error bars represent ±SEM.

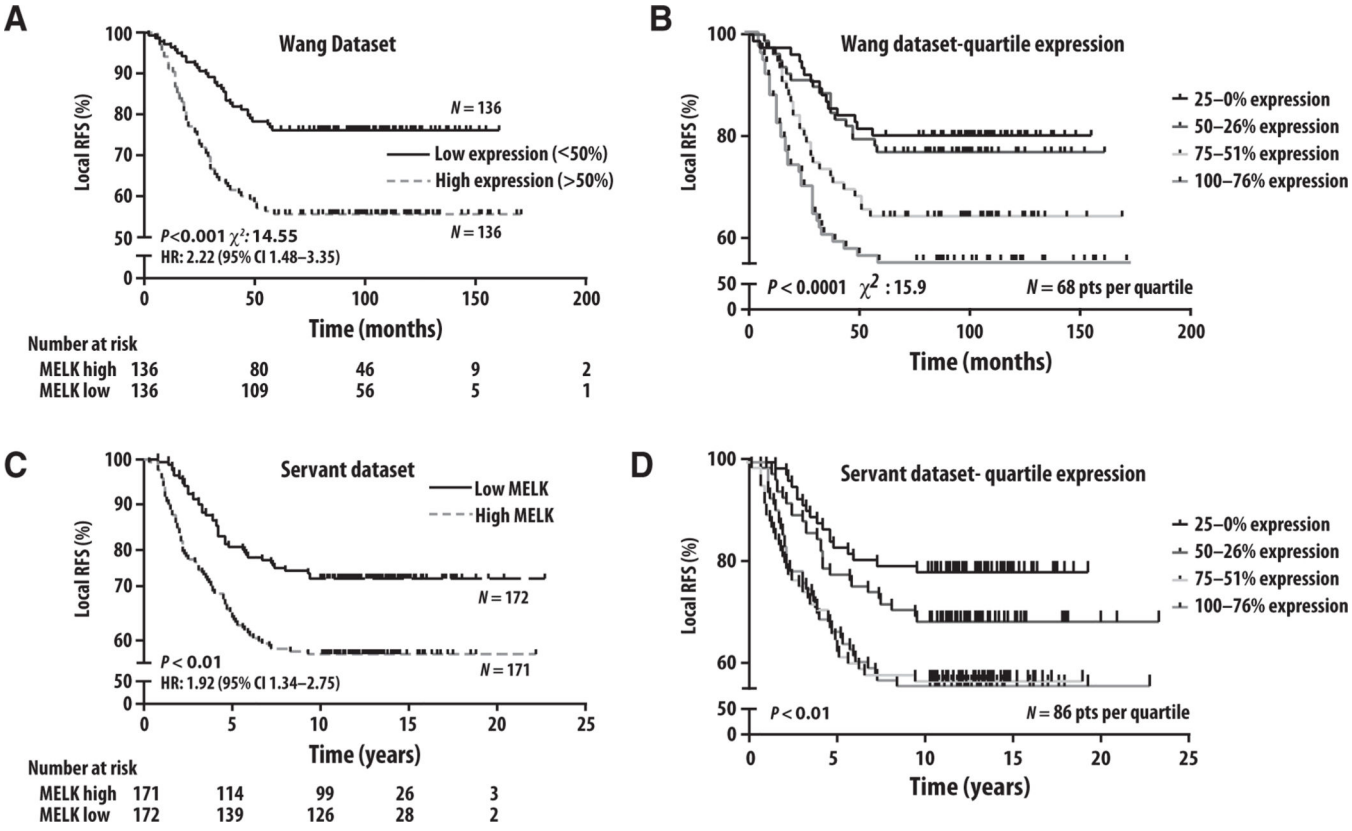

Figure 5.

MELK expression is associated with increased risk of local recurrence. Kaplan–Meier local RFS analysis in the Wang dataset demonstrates that patients whose tumors have higher than median expression of MELK have significantly higher rates of local recurrence after radiation and an overall poorer prognosis than patients with lower than median expression of MELK (HR for local recurrence, 2.22; $P < 0.001$; **A**). Even when divided into quartiles, increasing levels of MELK expression are associated with increased risk of locoregional (**B**). Similarly, in the Servant dataset, Kaplan–Meier local RFS analysis demonstrates that patients whose tumors have high expression of MELK have significantly higher rates of locoregional (**C**). Again, quartile expression of MELK demonstrates increasing rates of locoregional with increasing MELK expression (**D**).

Table 1.

Univariate and multivariable Cox proportional hazards regression analysis in Servant dataset

Univariate analysis[a]		
Covariate	**HR (95% CI)**	***P***
MELK expression	1.30 (1.10–1.60)	0.005
Age (increasing)	0.95 (0.92–0.98)	0.002
Grade	1.39 (1.07–1.82)	0.015
HER2 status	1.66 (1.07–2.58)	0.024
Molecular subtype	1.15 (1.02–1.29)	0.021
Multivariable Cox proportional hazards regression analysis [b]		
Covariate	**HR(95% CI)**	***P***
MELK expression (continuous)	1.28 (1.06–1.53)	0.01
Age (increasing)	0.96 (0.93–0.99)	0.01

NOTE: Univariate and multivariable analyses identifies MELK as the variable most strongly associated with local recurrence in patients with early-stage breast cancer treated with adjuvant radiation. Univariate analysis identifies several clinical and pathologic factors associated with local recurrence. In multivariable cox proportional hazards regression analysis of all patients, only MELK expression (continuous variable) remained significantly associated with worse local RFS. HRs and 95% CIs were calculated for all analyses and are listed.

[a]Chemotherapy, radiation dose, LVSI, ER status, PR status, nodal status, and T-stage. All nonsignificant.

[b]HER2 status, grade, and molecular subtype all nonsignificant of multivariable analysis.