The double life of hTERT: Identification of a novel function and an explanation of mechanism

Linda Zhou, Daniel Radin, Parth Patel, Donna Leonardi

Abstract— The goal of this experiment was to elucidate a novel telomere independent function of hTERT, the catalytic component of telomerase, involving the interplay between hTERT, the epidermal growth factor receptor and its ligand and hypoxia induced drug resistance. shRNA was used to knock down hTERT mRNA expression in MDA-MB-231, an aggressive triple negative breast cancer line. A modified Boyden chamber invasion assay showed that silencing hTERT significantly inhibited the cell's known positive chemotactic response to epidermal growth factor (EGF). An enzyme linked immunosorbant assay (ELISA) performed for evaluation of the activation of the EGFR showed that hTERT knockdown results in lower levels of total and phosphorylated EGFR independent of EGF ligand binding. In addition, the effect of silencing hTERT on hypoxia-induced cisplatin resistance in MDA-MB-231 cells was investigated. The MTS assay for cell viability demonstrated for hTERT knockdown cells, hypoxic conditions provided no resistance or proliferative advantage, with cisplatin demonstrating the same rate of effectiveness as in cells grown in normoxia, while hTERT positive cells show significant resistance to cisplatin in hypoxia. Timing of the experiment excludes telomerase activity as an explanation. Previous literature demonstrated that cisplatin resistance is related to EGFR activation, independent of EGF ligand binding. Thus, the identification of a relationship between hTERT and the EGFR supplements the original additional findings of this study that silencing hTERT mRNA expression can mediate hypoxia-induced cisplatin resistance independent of telomere length, suggesting both a possible treatment for drug resistant breast cancer tumors and a novel explanation for mechanism of action.

Index Terms— Cisplatin, EGF, EGFR, Hypoxia, hTERT, Normoxia, shRNA, Telomerase, Triple-Negative Breast Cancer

1 Introduction

A myriad of chemotherapeutic drugs are currently available as cancer treatments. While these drugs are initially very effective, an issue arises when tumors become drug resistant. It becomes increasingly difficult to treat cancer when individual cancer cells aggregate and form tumors, as tumors promote a hypoxic environment, which often results in the upregulation of cell survival proteins [10]. In particular, resistance against cisplatin, a last-resort type drug, routinely arises in triple-negative breast cancer tumors. This resistance is thought to be mediated by the hypoxia-inducible factor 1, alpha subunit (HIF1a), which is upregulated in hypoxia and greatly enhances a cascade of cell survival mechanisms. Hypoxia has been shown to promote proliferation across a spectrum of cell lines [11].

In this study, MDA-MB-231 cells were incubated in hypoxic conditions to simulate the chemical environment of a tumor. The observed hypoxia-induced cisplatin resistance was studied in the context of human telomerase reverse transcriptase (hTERT).

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hTERT is the catalytic subunit of telomerase, an enzyme that protects the telomeres of duplicating DNA strands and whose discovery was awarded the 2009 Nobel Prize in Medicine. For more than twenty years after the discovery of telomerase, scientists believed that the role of telomerase is limited to regulating telomere length [6]. However, recent research suggests that the subunits of telomerase have additional functions not related to regulating telomere length [15]. While these functions remain unclear, the hTERT subunit, which is exclusively detectable in cancerous cells, has been targeted as a possible mediator of telomere independent cellular pathways that promote cell survival and proliferation [3]. Silencing hTERT could thus be a potential anti-tumor treatment, as its exclusive presence provides a means of specifically targeting cancer cells.

In this study, hTERT mRNA expression in MDA-MB-231 cells was silenced using small hairpin RNA (shRNA), which is cleaved in the cell into small interfering RNA (siRNA) and ultimately results in knockdown of gene expression through the RNA interference pathway [11]. In addition to the effect of hTERT knockdown on drug resistance in hypoxia, an explanation of mechanism was also elucidated. Expanding on previous studies that report that there is a correlation between cisplatin resistance and activation of the epidermal growth factor receptor (EGFR) [2], the effect of hTERT knockdown on EGFR phosphorylation and chemotactic response to epidermal growth factor was examined. Chemotaxic migration is a marker of advanced cancers, and plays a key role in the metastasis of breast cancer tumors [9].

2 Procedure for Paper Submission

2.1 Cell Culture

MDA-MB-231 cells (ATCC No. HTB-26), a human breast adenocarcinoma cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). MDA-MB-231 cells overexpress the epidermal growth factor receptor (EFGR) and have upregulated hTERT mRNA expression [5]. Cells were maintained in RPMI-1640 medium supplemented with L-glutamine (Invitrogen, Calsbad California, USA) and 10% fetal bovine serum (Invitrogen). Cells were incubated at

37°C with 5% CO_2. The cell cultures were expanded when they reached near confluence utilizing 0.05% trypsin-EDTA (Invitrogen).

2.2 Transfection and Selection of Transformed Cells

MDA-MB-231 cells were transfected with either an hTERT shRNA plasmid or a non-effective GFP plasmid as a control. The plasmids contained an ampicillin and puromycin resistance sequence and were obtained from OriGene Technologies (Cat. No. TR32047, Rockville, MD). The plasmids were amplified in E.coli JM101 (Bio-Rad), and purified with a Maxiprep Kit (Invitrogen, K2100-17). Isolated plasmids were incubated with polyethyleneimine (PEI) (Sigma, 21195U) for 30 minutes in 100 uL serum free RPMI-1640 at a PEI: DNA ratio of 0.75 (w/w). MDA-MB-231 cells were plated in a 24-well plate at a density of 0.5 million cells/well. After 24 hours, the cultures were approximately 80% confluent and the cells were incubated with the DNA-PEI complex solution. The cells were replenished with new media after 24 hours and expanded into T25 culture flasks after 72 hours in puromycin selection media. To determine the optimal concentration of puromycin (Sigma, P8833) to use for selection, a puromycin kill curve was established. Untransformed MDA-MB-231 cells were plated at 100,000 cells/well in a 24-well plate. After 24 hours, the cells were exposed to puromycin concentrations ranging from 0 ug to 10 ug/mL, and cell viability were measured every day for four days. The minimum antibiotic concentration that killed 100% of cells in 4 days was selected as the optimal concentration. The transformed MDA-MB-231 cells were selected with 2 ug/mL puromycin for at least three weeks before harvesting for analysis.

2.3 Cell Proliferation Assay

The effect of hTERT knockdown on proliferative response to cisplatin was examined. Cells transfected with either an hTERT shRNA plasmid or with a non-effective GFP plasmid were seeded in 96-well plates at 12,000 cells/well in RPMI-1640 and 10% fetal bovine serum. The cells were incubated at 37°C with 5% CO_2 and in either normoxia (20% O_2) or hypoxia (3% O_2). After 24 hours, the cells were exposed to cisplatin (Sigma 479306) at concentrations ranging from 0 to 100 microM, or left as a control and incubated for an additional 24 hours. Cell viability was determined by using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). The assay was performed by adding 20 uL of the CellTiter 96® AQ_{ueous} One Solution Reagent to 100 uL off culture media and incubating the plate for 4 hours. To measure the conversion of tertrazolium to formazan product, the absorbance was recorded at 490 nm using a 96-well microplate reader (BioTek ELx808; BioTek, Winnoski, Vermont, USA).

2.4 Migration Assay

The effect of hTERT knockdown on cell migration in either hypoxia or normoxia was determined using a 96-well chemotaxis chamber assay (Chamber Series Neuroprobe, Cabin John, MD). Cells transfected with either an hTERT shRNA plasmid or with a non-effective GFP plasmid were plated in 400μL of RPMI-1640 in the upper wells of the chamber at a concentration of 40,000 cells/well, and the chemoattractant, recombinant human EGF, (400 μL/well) at 50, 100, or 200 ng/mL (Sigma) in the lower wells. The chamber was incubated for 12 hours in either hypoxia or normoxia. Cell migration was quantified by the number of cells that migrated directionally through a collagen I (40 mg/ml; Becton Dickinson, Bedford, MA)-coated 8-mm-pore polyvinyl pyrrolidone-free polycarbonate filter after 24 hours. After the incubation period, the filters were removed, fixed, and stained with Diff-Quik (Baxer Scientific, McGaw Park, IL) and mounted on glass slides. Nonmigrated cells were removed by wiping with a cotton swab on the opposite side of the filter. Triplicate wells were performed in each assay and ten random fields of vision/well (40x objective) were counted for quantification of cell migration [8].

2.5 Phosphorylated EGFR Assay

The effect of hTERT knockdown on the phosphorylation of the epidermal growth factor receptor was quantified using a cell based ELISA (KCB1095, R&D Systems, Minneapolis, Minnesota, USA). Cells transfected with either an hTERT shRNA plasmid or with a non-effective GFP plasmid were grown in 96-well plates and stimulated with EGF ligand. Following stimulation, cells are fixed and permeabilized in the wells with 4% formaldehyde (Sigma). The cells are simultaneously incubated with two primary antibodies: a phospho-specific antibody and a normalization antibody that recognizes the EGFR protein regardless of phosphorylation status. Two secondary antibodies recognizing the primary antibodies are labeled with either horseradish-peroxidase (HRP) or alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for either HRP or AP are used for detection. The fluorescence of the phosphorylated protein is normalized to that of the pan-protein in each well for the correction of well-to-well variations. Fluorescence is reported in relative fluorescence units (RFUs).

2.6 Telomerase Actvitiy Assay

The effect of hTERT knockdown on telomerase activity was measured via fluorescence using a PCR-based detection method called a Telomeric Repeat Amplification Protocol (TRAP) (S7707FR, Millipore, Massachusetts, USA) as per company assay instructions. Telomerase activity is reported in TPGs (Total Product Generated), where one unit corresponds to at least 3 telomeric repeats by telomerase in a 30 minute incubation at 30°C. Telomerase activity was measured three weeks after stable transformed cell lines were harvested.

2.7 Data Analysis

Data were analyzed using Microsoft Excel (Microsoft, Redmond, WA). All assays were repeated at least twice, with a sample size for each condition at least n=5. The unpaired Student's t-test was used to determine significance. Alpha value was set at 0.05. * refers to statistical significance between results of the control sample and the results of the indicated concentration *within* a cell line. * refers to statistical significance between the results of the Non-E-GFP control cells and the hTERT knockdown cells at the *indicated* concentration as well as a comparison between cells in normoxia vs. hypoxia at the indicated concentration.

3 RESULTS

Effect of cisplatin on cell viability in Normoxia

Cisplatin induces cell death in control MDA-MB-231 cells in a dose-dependent fashion ($p<0.05$). Cisplatin also induces cell death in hTERT knockdown cells in a dose-repsonse fashion ($p<0.05$). In normoxia, there is no significant resistance to cisplatin at higher concentrations.

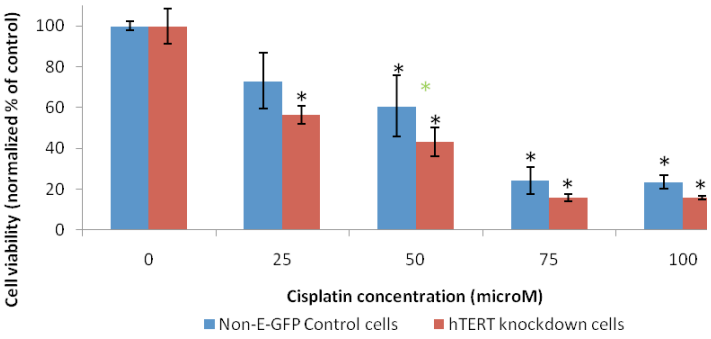

Fig 1. Effect of cisplatin on cell viability in normoxia. In normoxic conditions, there was no significant difference between cell viability of hTERT knockdown cells and control cells for all concentrations except at 50 microM. ($p > 0.05$) Both cells lines exhibit a dose-dependent response similar to that observed in previous literature. Data for each cell line was normalized to the results of the control (0 microM) for that cell line. Bars are means ± SD (n=3).

Effect of cisplatin on cell viability in Hypoxia

In order to determine cisplatin's oncolytic properties in hypoxia, cells were exposed to exogenous cisplatin treatment in hypoxia for 24 hours. Cisplatin was shown to have a higher efficacy on MDA-MB-231 cells when hTERT is knocked down. Cisplatin operates in a dose-response fashion for both cell lines, but a more exagerated dose-response is observed in hTERT knockdown cells at higher concentrations of 50-100uM ($p<0.05$).

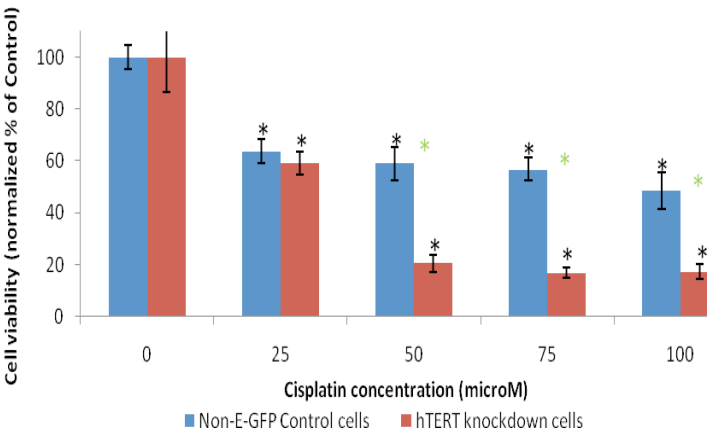

Fig 2. Effect of cisplatin on cell viability in hypoxia. In hypoxia, control cells show a significant resistance to cisplatin at 50, 75, and 100 microM when compared with hTERT knockdown cells. ($p < 0.05$) hTERT knockdown cells exhibit the same trend of dose-dependent response to cisplatin in hypoxia that was observed in normoxia. Data for each cell line was normalized to the results of the control (0 microM) for that cell line. Bars are means ± SD (n=3).

Effect of cisplatin on cell viability of control cells in hypoxic vs. normoxic conditions

Cisplatin administration produces a decline in cell viability in a dose-dependent response after 24 hours in normoxic and hypoxic conditions. It is interesting to note that cisplatin's oncolytic activity is significantly higher in normoxia at higher concentrations of 75 and 100 uM ($p<0.05$).

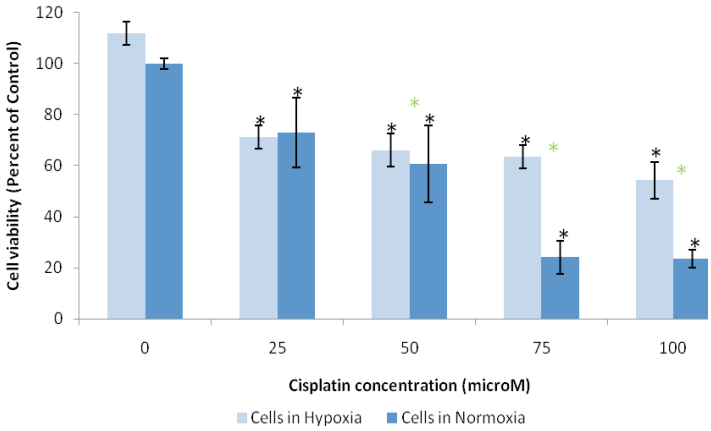

Fig 3. The effect of cisplatin on cell viability on control cells in hypoxia vs. normoxia. The data is normalized to that of the results obtained in normoxia (0 microM control). This accounts for the basal proliferative effect of hypoxic conditions on cell viability. Control cells show a dose-dependent response to cisplatin in normoxic conditions but show a significant resistance to cisplatin at 50, 75, and 100 microM in hypoxia. Bars are means ± SD (n=3).

The effect of cisplatin on cell viability of hTERT knockdown cells in hypoxic v.s. normoxic conditions

In hTERT knockdown MDA-MB-231, cisplatin induces cell death in a dose-response fashion in both normoxic and hypoxic conditions. The previously observed resistance of control cells to cisplatin in hypoxia is not observed when hTERT is knocked down using shRNA.

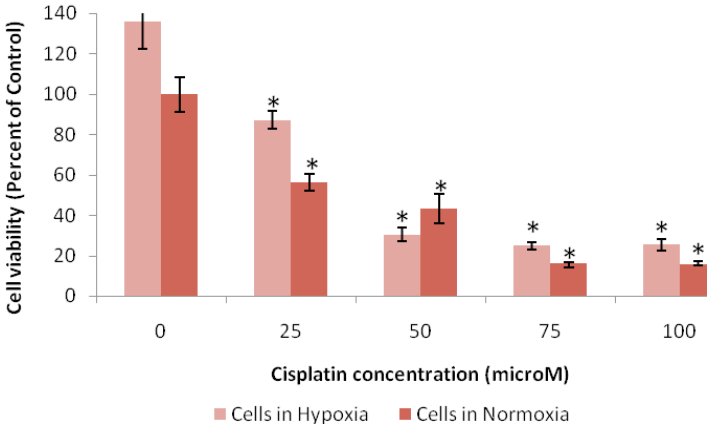

Figure 4. Effect of cisplatin on viability of hTERT knockdown cells in hypoxia vs. normoxia. The data is normalized to that of the results obtained in normoxia (0 microM control). This accounts for the basal proliferative effect of hypoxic conditions on cell viability. hTERT knockdown cells exhibit a dose-dependent response to cisplatin in both normoxic and hypoxic conditions. Bars are means ± SD (n=3).

Effect of hTERT on cell migration to EGF

A migration assay was performed in the presence of epidermal growth factor to determine whether hTERT levels modulate the cell's migratory response. EGF stimulated a strong migratory response in control cells, with almost 300% more cells migrating in response to the highest concentration of EGF than in the control group. There was a significant dose-dependent migratory response toward 50, 100 and 200 ng/mL EGF when compared to the blank control ($p<0.05$). In hTERT knockdown cells, the migratory response is modulated with only 126% of cells in the presence of 200ng/mL EGF ($p<0.05$). There was no significant migration at 50 or 100ng/mL EGF in the hTERT knockdown cells.

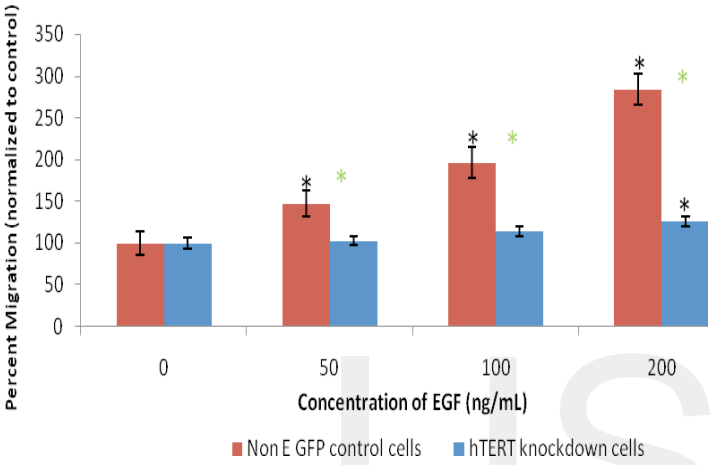

Fig 5. Effect of hTERT on cell migration to EGF. Control cells exhibited more migration to EGF at all concentrations when compared to hTERT knockdown cells. Bars are means ± SD (n=3).

Effect of hTERT knockdown on EGFR activation

EGFR activation was evaluated in order to determine whether varying hTERT levels in this cancer cell type play a role in EGF mediated phosphorylation of EGFR. An enzyme linked immunosorbant assay was run for both basal phosphorylation and EGF induced phosphorylation of EGFR. There was no statistically significant difference between basal phosphorylation of EGFR in hTERT knockdown cells and in control cells. However, in the presence of 12.5, 50, and 100 ng/mL EGF hTERT knockdown cells exhibited significantly lower levels of phosphorylated EGFR than control cells ($p< 0.05$). For both hTERT knockdown and control cells, there was no significant dose-dependent response.

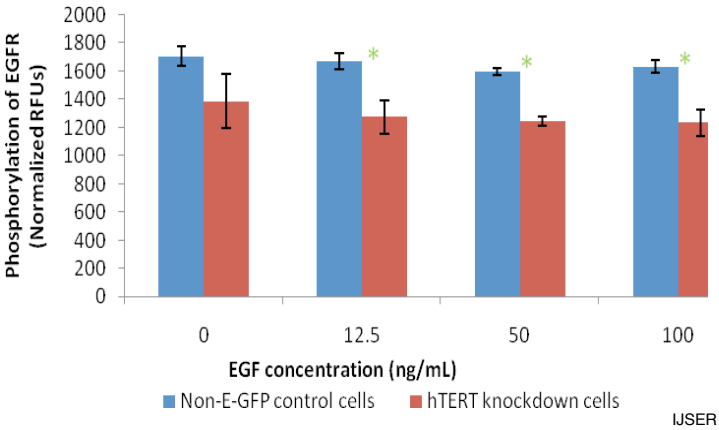

Fig 6. Effect of hTERT knockdown on EGFR phosphorylation. In the presence of EGF, hTERT knockdown cells exhibit less EGFR phosphorylation. Bars are means ± SD (n=3).

Effect of hTERT knockdown on EGFR expression

An enzyme linked immunosorbant assay was run for both basal phosphorylation and EGF induced phosphorylation of EGFR. There was no significant difference between basal total EGFR expression in hTERT knockdown and control cells. However, in the presence of 50 and 100 ng/mL EGF, hTERT knockdown cells exhibited significantly less total EGFR expression. For both hTERT knockdown and control cells, EGFR expression was not dose-dependent.

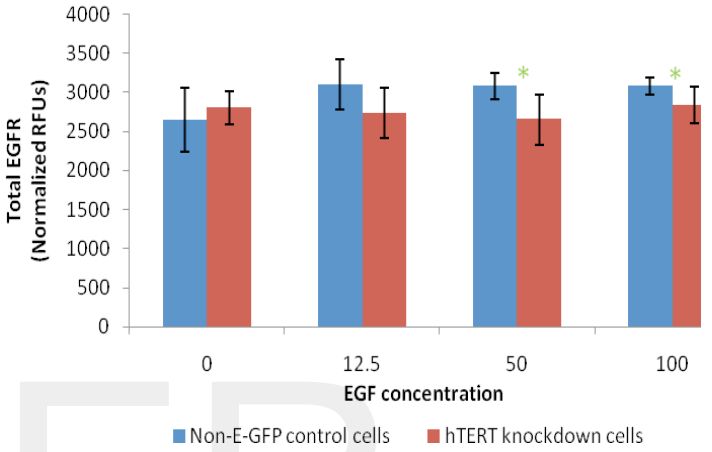

Fig 7. Effect of hTERT on global EGFR levels. Control cells exhibited more total EGFR at high concentrations of EGF when compared to hTERT knockdown cells. Bars are means ± SD (n=3).

Telomerase activity in knockdown vs. control cells

Telomerase activity was measured in knockdown cells and control cells while simultaneously examining telomerase activity in the internal control containing cells with spiked telomerase. Results indicate that the shRNA against hTERT significantly reduced telomerase activity in MDA-MB-231 ($p<0.05$).

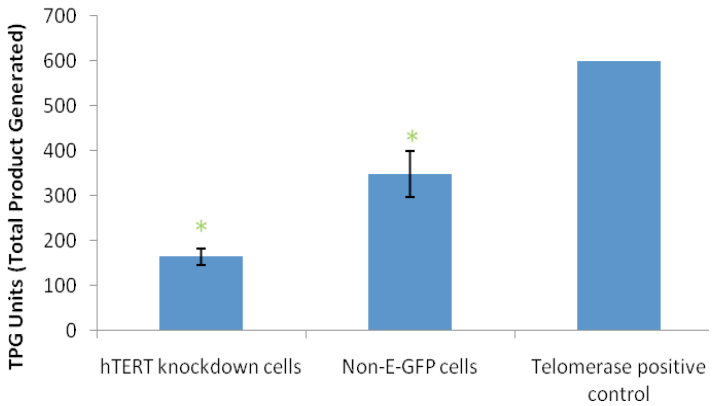

Fig 8. shRNA against hTERT reduces telomerase activity. hTERT knockdown cells displayed only half the telomerase activity relative to the cells transformed with the control plasmid. This is a significant decrease ($p<.05$). The telomerase positive control corresponds to the telomerase activity of 1000 cells spiked with telomerase extract. Bars are means ± SD (n=3).

4 Discussion

This study suggests for the first time that silencing hTERT mRNA expression can mediate hypoxia-induced cisplatin resistance in the most aggressive form of breast cancer independent of telomere length. Furthermore, this study identified a novel relationship between hTERT, chemotactic response to EGF, and EGFR phosphorylation that could give insight to currently obscure regulatory mechanisms of hTERT.

In this study, breast cancer cells were incubated in a hypoxic environment to simulate tumor conditions. As expected, while the control cells showed a dose-dependent death curve in response to cisplatin in normoxia, there was a significant resistance to cisplatin in hypoxia. However, knockdown of hTERT nulled the advantages of hypoxia that MDA cells exploit. hTERT knockdown cells exhibit the same dose-dependent response to cisplatin in hypoxia observed in normoxia with no indication of resistance. It is interesting to note that hTERT knockdown in *both* normoxia and hypoxia significantly decreased the survival of breast cancer cells compared to control cells, with an enhanced decrease observed in hypoxia.

While differences in cell proliferation in general can be attributed to shortened telomeres as a result of hTERT knockdown (displayed in Figure 8), the *absence of resistance* to cisplatin *in hypoxia* must be independent of telomere length and regulation because the results were observed after only 24 hours of hypoxic incubation, before telomeric changes can occur in cancer cells [6]. This suggests that the knockdown of hTERT itself induced these effects. This, in turn, suggests a masked regulatory function of hTERT that only becomes evident in hypoxia. The next step was to investigate this function, and how hTERT knockdown mediates hypoxia induced cisplatin resistance.

It has been shown that cells phosphorylate the EGFR as a survival response to toxic levels of cisplatin, and this activation reduces efficacy of cisplatin treatment [2]. Interception of EGFR activation has thus been identified as a useful strategy for overcoming cisplatin resistant tumors. This study therefore investigated modifications in EGFR activity in conjunction with hTERT knockdown as a possible explanation for mechanism of action.

MDA-MB-231 cells overexpress EGFR [5]. Previous literature found that when MDA cells are treated with EGF, this overexpression correlates to an increase in migration but not proliferation [8]. This result was reproduced in this study. Treating MDA cells (transfected with a non-effective GFP plasmid, used as a control) resulted in no significant proliferation across various concentrations of EGF. However, control cells showed a significant dose-dependent chemotactic migratory response to EGF, culminating in an almost 300% increase in migration at the highest concentration (200 ng/mL). This migratory response was mediated in hTERT knockdown cells, with only 120% increase in migration of cells moving towards the highest EGF concentration. These results indicate a regulatory function of hTERT that involves EGF ligand binding which culminates in migration. An additional phenomena was observed: control cells migrated and aggregated in the presence of EGF, while transformed hTERT knockdown cells displayed no aggregation after migration (pictures not shown). This could be a result of the low number of hTERT knockdown cells that migrated, or of hTERT knockdown-induced downregulation of cell aggregation factors and receptors [13]. Further research is required in this area.

While hTERT was shown to regulate cellular response to EGF, the results of the phosphorylated EGFR assay indicate that this response does not involve the EGF receptor and that there is instead an additional function of hTERT regulating EGFR activation and expression. hTERT knockdown cells exhibit significantly less activated EGFR than MDA cells transfected with the control plasmid. This EGFR activation was independent of the concentration of EGF present. Because there was no significant difference between phosphorylation of the EGF receptor across the concentrations of EGF, this implies that the cellular response to EGF culminating in migration is independent of the EGF receptor and involves the chemotactic response of another receptor. This study thus demonstrates two previously unreported functions of hTERT; in addition to regulating cell migration through an EGFR independent chemotactic response pathway, hTERT also activates the EGF receptor independently of ligand binding activity.

These results further support a relationship between hTERT mRNA level and cisplatin resistance. Knockdown of hTERT mRNA diminished cisplatin resistance in hypoxia and resulted in lower levels of both phosphorylated and total EGFR; current literature concludes that a cell with less phosphorylated EGFR shows decreased resistance to cisplatin. Additionally, studies state that cisplatin induced EGFR activation is also independent of EGF ligand binding [12]. This suggests that overexpression of hTERT facilitates hypoxia induced cisplatin resistance via an EGFR related pathway.

It is important to consider the results of this study in the context of a hypoxic environment. Reports show that the HIF1a gene upregulates both the EGFR and hTERT expression [7], [14]. In a cancer cell, this corresponds to the increased proliferation seen when cancer cells form tumors. In this study, hTERT knockdown cells had no survival advantage in hypoxia, suggesting that hTERT is a major upstream regulator of the HIF1a induced cell survival response. Additionally, upregulation of EGFR in hypoxia is consistent with the emergence of cisplatin resistance in hypoxia. Thus, it is possible that in hypoxia, the relationship between hTERT and cisplatin resistance is magnified because of both increased levels of hTERT, and increased expression of EGFR, meaning there are more EGFR molecules available to phosphorylate.

Future research will involve specifying the relationship between hTERT, cellular response to EGF, activation of the EGFR, and HIF1a. Coupling the results presented in this paper with previous literature suggests that hTERT has a function regulating the MAPK signaling pathway [1], particularly ERK signaling. This connection is currently being researched for further development of this research project.

5 Conclusion

The results of this study show for the first time that silencing of hTERT mRNA expression inhibits activation of the EGFR in breast cancer cells. Additionally, this inhibition is independent of EGF ligand binding. The results of the migration assay show that hTERT has an additional, previously unrecorded function related to chemotactic response to EGF, and involving receptors other than the EGFR. The identification of a relationship between hTERT and the EGFR supplements the original additional findings of this study that silencing hTERT mRNA expression can mediate hypoxia-induced cisplatin resistance independent of telomere length, suggesting both a possible treatment for drug resistant breast cancer tumors and a novel explanation for mechanism of action.

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