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Coordinated Regulation of β-Tubulin Isotypes and Epithelial-to-Mesenchymal Transition Protein ZEB1 in Breast Cancer Cells

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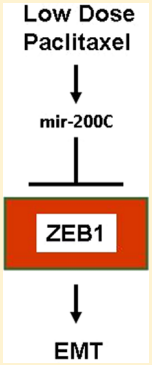
ABSTRACT: The regulation of β-tubulin isotypes, the primary targets for antimitotic chemotherapeutic drugs like taxanes, has implications for drug response and drug resistance. Over the past 15 years, micro-RNAs have been studied widely as regulators of mRNA levels. For example, the tumor suppressor miR-200c was shown in cell culture to target mesenchymal genes, including ZEB1 [Cochrane et al. (2009) *Mol. Cancer Ther. 8* (5), 1055−1066]. In that work, exogenous miR-200c was also shown to reduce β-tubulin class III, one of its predicted targets. Furthermore, decreased miR-200c and increased β-tubulin class III were associated with poor outcomes for ovarian cancer patients [Cittelly, D.M. et al. (2012) *Mol. Cancer Ther. 11* (12), 2556−2565]. Because miR-200c targets the epithelial-to-mesenchymal inducer ZEB1, we wanted to know whether changes in ZEB1 parallel β-tubulin isotype changes, implicating β-tubulin isotypes in ZEB1-associated cell survival pathways. We found coordinated positive feedback regulation of mRNA for ZEB1 and β-tubulin isotype classes I, III, and IVB in MDA-MB-231 breast cancer cells, commonly used as a model for triple-negative breast cancers. Low levels of paclitaxel (40 nM) were found to significantly reduce mRNA levels for these tubulin genes along with a 2−3-fold increase in miR-200c. ZEB1 silencing also reduced β-tubulin isotype classes I, III, and IVB mRNA, whereas upregulation of ZEB1 was associated with increases in these isotype classes. Our work indicates that paclitaxel-induced reduction of ZEB1 and β-tubulin isotypes are, in part, due to increased activity of miR-200c. These results suggest that in aggressive breast cancers, as modeled by MDA-MB-231 cells, β-tubulin class III is a biomarker for cell survival mediated through ZEB1-induced tumor progression pathways.



Taxanes, commonly used in chemotherapy protocols for breast cancer and other solid tumors, target β-tubulin isotypes. Eight genes code for different β-tubulin isotypes, and the protein products are grouped into seven isotype classes based upon their carboxyl terminal sequence. Understanding the regulation of these genes is critical for explaining mechanisms that link this complex family of proteins to tumorigenesis and drug response. Over the past fifteen years, micro-RNAs have been shown to regulate mRNA degradation or protein translation.[1−3] Much interest has focused on the miR-200 family, in particular miR-200c, which was shown to degrade β-tubulin isotype class III (*TUBB3*) mRNA.[4,5] In a study of poorly differentiated, aggressive endometrial, breast, and ovarian cancer cells, Cochrane et al. (2009) demonstrated that the addition of exogenous miR-200c to cancer cells reduced mesenchymal proteins and their targets, as well as predicted miR-200c targets such as β-tubulin class III.[4] Furthermore, their work showed that treatment of aggressive ovarian cancer cells (HEC50) that normally express very low levels of miR-200c with pre-miR-200c increased the sensitivity of cells to vincristine, Epothilone B, and paclitaxel while reducing β-tubulin isotype class III levels.

The importance of β-tubulin class III as a cancer biomarker has long been of interest. It reduces the stability of microtubules and increases their dynamic instability.[6] Because taxanes, like paclitaxel, stabilize microtubules in interphase cells and in mitotic spindles, increases in β-tubulin class III are thought to contribute to drug resistance both in cell cultures and in vivo. Leskela et al. (2011) suggested that in ovarian tumors, levels of β-tubulin class III are regulated by miR-200c.[5] Their work demonstrated that in clinical samples from ovarian tumors, low miR-200c levels were associated with high β-tubulin class III protein and patients whose tumors had low miR-200c levels had a poorer response to treatment, indicating that this protein and miR-200c are clinically significant biomarkers.[5] Using an inducible system to increase miR-200c levels in ovarian cancer Hey cells and in mouse tumor xenografts, Cittelly et al. (2012) showed that increasing miR-200c sensitized cells to paclitaxel and correlated with a reduction in β-tubulin class III.[7]

A major target of miR-200c is the zinc finger transcription factor ZEB1. The reciprocal regulation of miR-200c and its target ZEB1 plays a role in tumor metastasis and cell invasion for many types of cancers, including breast cancer.[8] Upon binding to DNA, ZEB1 has been shown to act as a transcriptional activator or repressor.[9] It is a potent inducer of cell morphogenesis through epithelial-to-mesenchymal (EMT) pathways and suppresses miR-200c transcription, leading to increases in miR-200c targets.[8] In addition, ZEB1 mRNA is a major target of miR-200c. The EMT cell phenotype is characterized by the loss of polarity and cell adhesion proteins, such as claudins and cadherins.[10] This reduction in cell anchoring proteins leads to invasion and metastasis. The work of Ferlini and colleagues suggests that β-tubulin class III is also a survival factor, and their work directly links β-tubulin class III protein to the activity of GTPases (GBPI and GNAII) that contribute to tumor cell survival in oxygen-poor microenvironments.[9] Because miR-200c was shown to regulate β-tubulin isotype class III, it is possible

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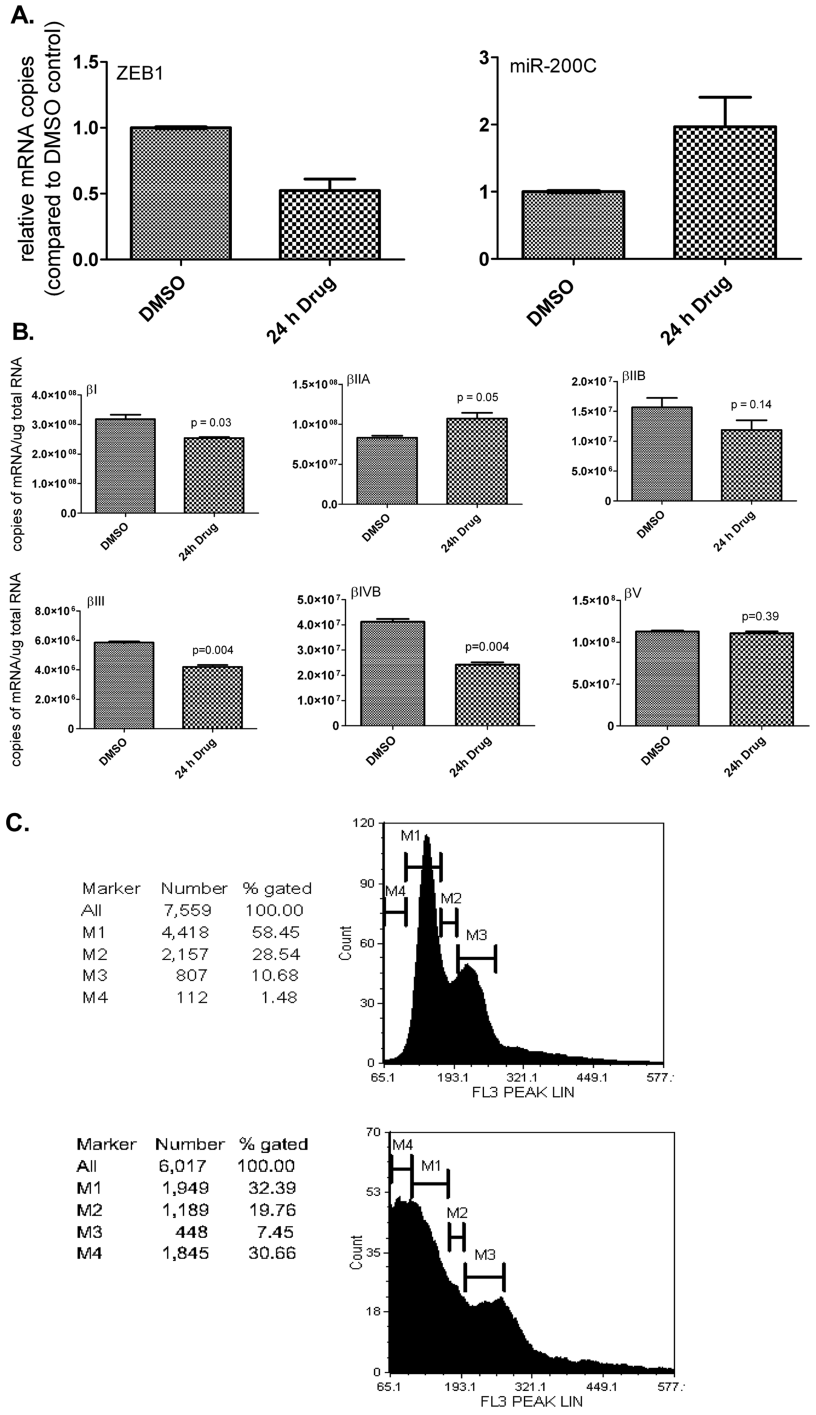

Figure 1. qRT-PCR for ZEB1, miR-200c, and β-tubulin isotypes after paclitaxel treatment of MDA-MB-231 cells. MDA-MB-231 cells were treated with 40 nM paclitaxel for 24 h. Panel A. Left bar graph: Quantitative RT-PCR using a standard curve and normalizing to 1 μg total RNA was used to measure ZEB1 in MDA-MB-231 cell samples. The relative amount of ZEB1 mRNA was calculated by dividing the mRNA number of copies for the drug-treated samples by the number of copies for the DMSO-treated samples. The mean relative amount for two independent cell preparations is shown, and the error bars represent the standard deviations. Right bar graph: Comparative RT-PCR was used to measure changes in miR-200C in MDA-MB-231 cells relative to those with no drug controls, using U6 and SCA17 as housekeeping genes for normalization. Error bars for miR-200C represent the standard deviations of duplicate experiments with two independent cell cultures. Panel B. Quantitative RT-PCR using a standard curve and normalizing to 1 μg total RNA was used to measure β-tubulin isotypes in MDA-MB-231 cells. The error bars represent the standard deviations from duplicate samples. The data are representative of three independent cell preparations. The statistical significance for Student *t* tests is shown above the bar graphs, and the level of statistical significance was set at $p = 0.05$. Panel C. Flow cytometry of MDA-MB-231 cells treated with 40 nM paclitaxel for 24 h and stained with propidium iodide showing the distribution of cells in the cell cycle. M1 = G1 phase, M2 = S phase, M3 = G2/M phase, and M4 = hypodiploid. Upper diagram: control cells. Lower diagram: paclitaxel-treated cells.

that pathways involving ZEB1 also contribute to mechanisms regulating β-tubulin class III, supporting the notion that this tubulin isotype is a biomarker for both tumor cell survival and cancer progression.

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Because ZEB1 and β-tubulin isotype class III mRNA are major targets for miR-200c, we hypothesized that changes in β-tubulin class III might parallel changes in ZEB1. In the work described here, we show that the expression of β-tubulin classes I, III, and IVB in MDA-MB-231 breast cancer cells is closely associated with the expression of EMT protein ZEB1. A reduction in these β-tubulin isotype classes and ZEB1 can be induced by low concentrations of paclitaxel. Our work shows that paclitaxel treatment reduces ZEB1 and β-tubulin classes I, III, and IVB mRNA in part by increasing miR-200c levels. MDA-MB-231 breast cancer cells lack receptors for HER2, estrogen, and progesterone and are commonly considered a cell culture model for aggressive breast cancers that are often treated with chemotherapy protocols including taxanes. Our data indicate that this close association between ZEB1 and β-tubulin isotype mRNA is in part due to regulation by miR-200c. Because of this association, we hypothesize that changes in signaling pathways involving ZEB1 may contribute to paclitaxel resistance.

MATERIALS AND METHODS

Cell Culture and Paclitaxel Treatments. MDA-MB-231 and MCF7 cells were from American Type Culture Collection (Manassas, VA) and were grown at 37 °C and 5% CO_2 according to the supplier's recommendations. Cells were grown in 75 cm^2 flasks for real time PCR experiments or in six-well plates for transfection experiments. When cells reached 60−80% confluence, they were treated with 40 nM paclitaxel in DMSO or DMSO (control cells) for 24, 48, or 72 h. Cells were cultured independently a minimum of three times for all experiments.

Real Time qRT-PCR. We used a two-step qRT-PCR to determine the amount of mRNA present for each β-tubulin isotype (classes I, IIA, IIB, III, IVB, and V). Total RNA was extracted and qRT-PCR experiments were carried out using primers and protocols described previously.[12] qRT-PCR was performed on duplicate samples using SYBR Green I (Invitrogen, Carlsbad, CA) as the detection method with a Stratagene Mx3000P real time PCR instrument (Agilent Technologies, La Jolla, CA). The amount of β-tubulin isotype mRNA in a known amount of total RNA, determined by A_{260} measurements, was calculated using standard curves or using comparative RT-PCR with two or more housekeeping genes for normalization. Comparative PCR data were analyzed using the $\Delta\Delta C_t$ method.[12] Our primers for the tubulin isotype (classes I, IIA, IIB, III, IVB, and V) have been previously reported.[13] The primers used in this work for ZEB1 were as follows:

Forward Primer: CCGCGGCGCAATAACGTTACAA

Reverse Primer: GCCCTTCCTTTCCTGTGTCATCCTC

For micro-RNA experiments, qPCR assays (Qiagen, Valencia, CA) were used to measure miR-200c levels in cell samples according to the manufacturer's instructions. Comparative RT-PCR was done using miScript II RT kits and miScript Primer assays (Qiagen, Valencia, CA) with SCA17 (Hs_SCARNA17_1) and U6 (Hs_RNU6B_2) as housekeeping genes.

Western Blotting. Western blotting was done as described previously.[13] The monoclonal antibodies were DM1A for α-tubulin (Sigma Aldrich, St. Louis, MO), SAP4G5 (Sigma Aldrich, St. Louis, MO) for β-tubulin class I, and MAB374 (Millipore, Temecula, CA) for GAPDH. The ZEB1 antibody (ARP32422) was from Aviva Systems Biology (San Diego, CA).

Transfection Experiments (ZEB1, miR-200c, and ZEB1 siRNA). For miR-200c and ZEB1 siRNA transfection experiments, cells were transferred to six well plates at a density of 100 000−200 000 cells per well. ZEB1 and miR-200c transfections were carried out using Silencer siRNA transfection kit or Pre-miR miRNA Precursor Starter Kit (Ambion, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. After 24 h, the medium was replaced with fresh medium containing 40 nM paclitaxel or medium with an equivalent amount of DMSO for control cells (treated with a scrambled micro-RNA sequence). Total RNA for micro-RNA experiments was extracted as described previously.[12]

For overexpression of ZEB1, a TrueClone plasmid expressing human ZEB1 transcript variant 1 (accession number NM_001128128.1) (OriGene, Rockville, MD) was used according to the manufacturer's instructions. Cells were transfected transiently using Fugene HD (Promega, Madison, WI) with the vector containing ZEB1 or the empty vector in control experiments. MCF7 cells in were plated (six-well plates) at a density of 6.5×10^5 cells/well and grown to 10% confluency (4 days). Cells were harvested 48 or 72 h after transfection. In other experiments, MCF7 cells were grown in 25 cm^2 flasks, transfected at 10% confluency, and harvested at 48 or 72 h. MDA-MB-231 cells in were plated (six-well plates) at a density of 2.3×10^5 cells/well and grown to 50% confluency (3 days). Cells were harvested 24 h after transfection.

Paclitaxel and Cycloheximide Experiments for Protein Stability. When cells reached the log phase of growth (80% confluence), cells were treated with 10 μM cycloheximide for 24 h. Then media were removed and cells were treated with media plus DMSO, cycloheximide, 40 nM paclitaxel, or cycloheximide plus paclitaxel for 24 h. Cells were pelleted and used for Western blots.

Flow Cytometry. MDA-MB-231 cells were grown to 60−70% confluence and treated for 24 h with DMSO or 40 nM paclitaxel. Cells were washed with PBS and fixed with 70% ethanol. Just prior to scanning by flow cytometry (Gallios, Beckman Coulter, Inc., Indianapolis, IN), cells were washed twice with PBS and stained with propidium iodide (20 μg/mL) for 30 min. Duplicate experiments were analyzed using FCS Express version 3 (De Novo Software, Los Angeles).

RESULTS

Paclitaxel Treatment Reduces ZEB1 and β-Tubulin Isotype mRNA Levels and Increases miR-200c Levels in MDA-MB-231 Cells. Prior work in our lab showed that high doses of paclitaxel (400 nM) altered levels of several micro-RNAs, including miR-200c, and β-tubulin isotype mRNA in MCF7 breast cancer cells.[14] We wanted to know whether paclitaxel also induced changes in miR-200c levels in MDA-MB-231 breast cancer cells that are used as a model for aggressive basal-type triple negative breast cancer. We treated MDA-MB-231 cells with 40 nM paclitaxel for 24 h and used qRT-PCR to measure miR-200c levels and ZEB1 mRNA levels. We found that drug treatment reduces ZEB1 mRNA copy number by 50% relative to cells treated with the drug vehicle DMSO (Figure 1A, left panel). On average, there was a 2-fold increase in miR-200c (Figure 1A, right panel). These results are consistent with the reciprocal relationship between ZEB1 mRNA and miR-200c that has been previously reported.[15]

Because miR-200c is reported to regulate β-tubulin class III,[4] we used qRT-PCR to measure β-tubulin isotype classes I, IIA, IIB, III, IVB, and V in the presence and absence of exposure to 40 nM paclitaxel for 24 h. We found a significant reduction in β-tubulin isotype classes I, III, and IVB (Figure 1B). Flow cytometry measurements indicate that the 40 nM paclitaxel treatment increased the hypodiploid fraction (Figure 1C). Thus,

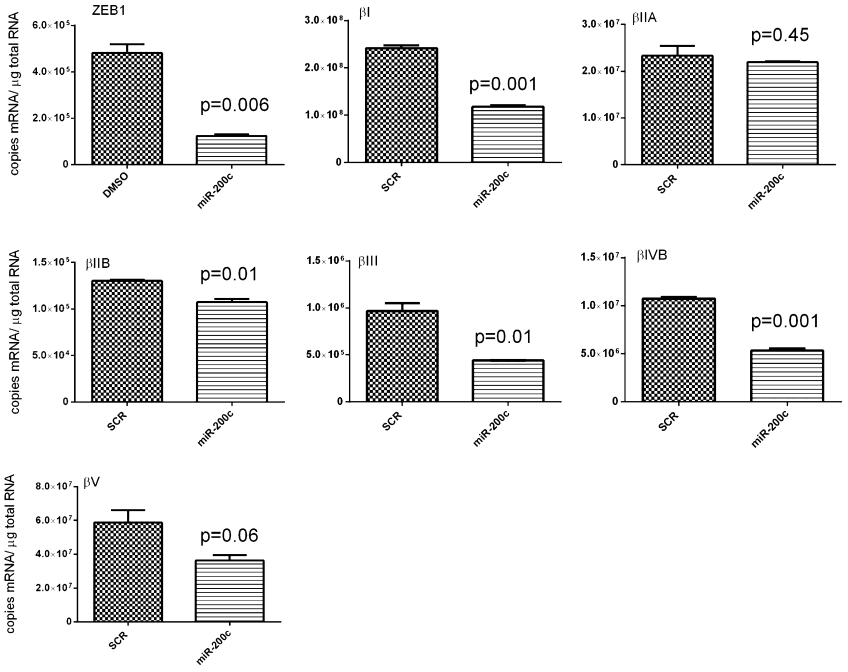

Figure 2. Transfection of MDA-MB-231 cells with miR-200c. Quantitative RT-PCR data were analyzed using standard curves for each β-tubulin isotype, and mRNA copies were normalized to 1 μg of total RNA. (SCR = scrambled sequence control.) Experiments were done with triplicate individual cell cultures. A representative experiment is shown. The error bars represent the standard deviation from duplicate samples from a single experiment. The statistical significance for Student t tests is shown above the bar graphs, and level of statistical significance was set at $p = 0.05$.

the decreases in ZEB1 and β-tubulin isotype mRNA are not associated with cell cycle block in G2/M.

Transfection of MDA-MB-231 Cells with miR-200c Reduces ZEB1 and β-Tubulin Isotype mRNA. miR-200c is in low abundance in untreated MDA-MB-231 cells. Typically the threshold cycle (Ct) for miR-200c is 35 or greater. To determine whether miR-200c can regulate mRNA for β-tubulin isotypes in MDA-MB-231 cells, we transfected cells with miR-200c (pre-hsa-miR-200c) and measured β-tubulin isotype mRNA. We found that ZEB1 and β-tubulin isotype classes I, IIB, III, IVB, and V were all significantly reduced by pre-hsa-miR-200c treatment relative to the scrambled sequence control (Figure 2).

ZEB1 Silencing Is Associated with Significant Reductions in β-Tubulin Isotype mRNA. miR-200c levels increased 400−1000-fold with transfection of miR-200c into MDA-MB-231 cells relative to the scrambled sequence control. This was considerably higher than the 2-fold increase in miR-200c found with paclitaxel treatment. Because the levels of miR-200c were significantly higher in the miR-200c transfected cells than in the paclitaxel-treated cells, we wanted to determine whether reduction in ZEB1 mRNA levels would result in smaller increases in miR-200c and also changes in β-tubulin isotype mRNA levels. We used siRNA technology to silence ZEB1 on average by 50% in MDA-MB-231. We found that this reduction in ZEB1 increased miR-200c levels 2−3-fold compared to the levels of the scrambled sequence controls in the presence or absence of 24 h treatment with 40 nM paclitaxel (Figure 3A). In addition, β-tubulin isotype mRNA for classes I, III, and IVB were all significantly reduced (Figure 3B). We also found a small but significant change in β-tubulin class IIA mRNA in these experiments. Thus, small increases in miR-200c and a 50% reduction in ZEB1 mRNA are associated with a reduction in mRNA for these β-tubulin isotypes. These small changes can be induced by 24 h, 40 nM paclitaxel treatment. Interestingly, β-tubulin class V mRNA was unchanged by ZEB1 silencing.

Upregulation of ZEB1 in MDA-MB-231 Cells Is Associated with Increased β-Tubulin Isotype mRNA. Given the correlation between ZEB1 levels and the mRNA levels for β-tubulin isotypes, we hypothesized that an increase in exogenous ZEB1 mRNA would be associated with an increase in β-tubulin isotype mRNA. We transfected MDA-MB-231 cells with a TrueORF vector pCMV6-Entry containing ZEB1. Empty vectors were used as controls. We found that a 3-fold increase in ZEB1 was associated with a significant 1.5−4-fold increase in mRNA for β-tubulin classes I, IIB, III, IVB and V (Figure 4). β-tubulin class IIA mRNA levels increased less than 1.5-fold.

In order to confirm the correlation between ZEB1 levels and β-tubulin isotypes levels, we carried out the same experiments with MCF7 cells, another breast cancer cell line known to have reduced levels of endogenous ZEB1 with increased levels of miR-200c compared to the levels of MDA-MB-231 cells. Figure 5A shows that MCF7 breast cancer cells have about 1000-fold fewer ZEB1 mRNA than MDA-MB-231 cells. When MCF7 cells were transfected with ZEB1, we achieved an increase in ZEB1 mRNA from 3-fold to as high as 30-fold. β-tubulin mRNA levels all increased significantly (2−10-fold) in these experiments. Figure 5B shows an example where β-tubulin isotypes increased 5−10-fold. Figure 5C shows that miR-200c was reduced significantly by transfection of ZEB1 into MDA-MB-231 and MCF7 cells. In summary, an increase in ZEB1 by exogenous transfection of MDA-MB-231 or MCF7 cells leads to significant parallel changes in β-tubulin classes I, IIB, III, IVB, and V mRNA, and these changes are linked to reciprocal changes in miR-200c.

ZEB1 Protein and mRNA Levels Are Correlated. To determine whether the loss of ZEB1 mRNA is associated with changes in ZEB1 protein, the effects of 24 h paclitaxel treatment on ZEB1 protein levels were measured. The results show that ZEB1 protein levels were unaffected in spite of decreased mRNA levels (Figure 6A).

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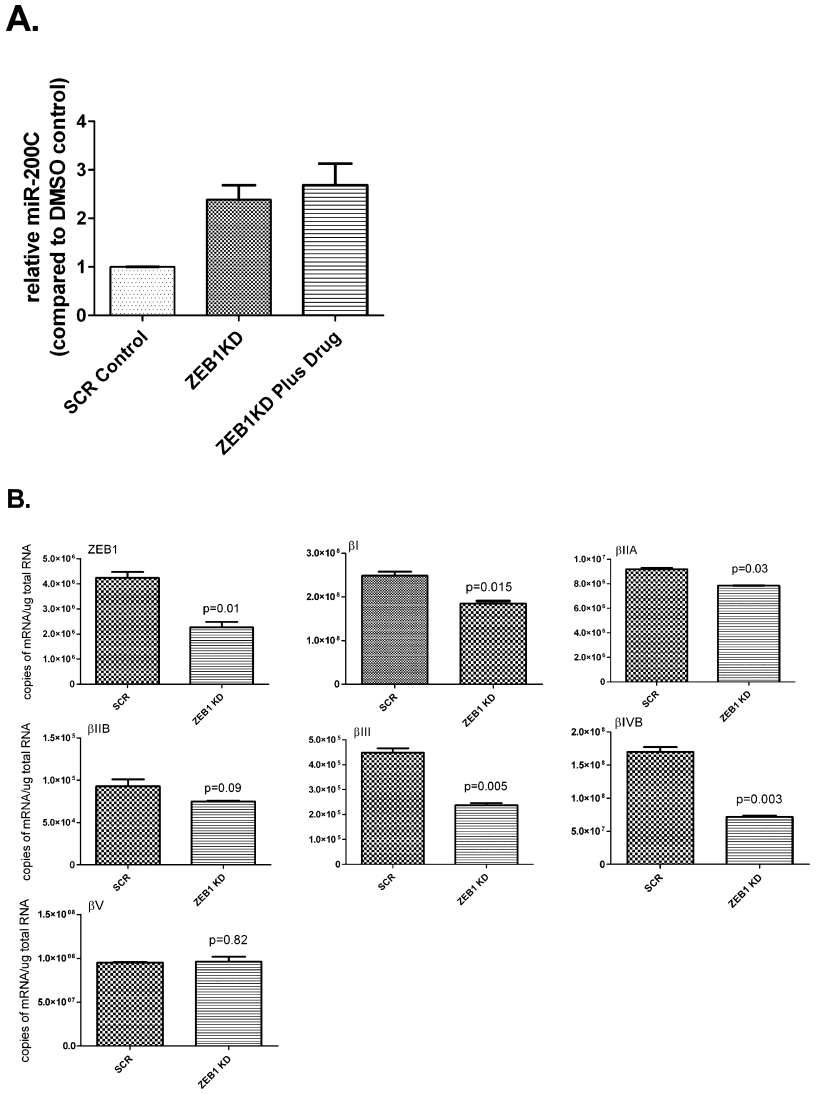

Figure 3. ZEB1 silencing in MDA-MB-231 cells using siRNA interference. Panel A. Comparative PCR was used to measure miR-200C using U6 and SCA17 as housekeeping genes for normalization. The error bars represent the standard deviations from duplicate experiments. The data for the ZEB1-silenced (ZEB1KD) and ZEB1-silenced plus 40 nM paclitaxel are shown relative to the DMSO control transfected with a scrambled sequence and set equal to 1. The relative silencing of ZEB1 in this experiment is shown to be 50% in panel B. Panel B. Quantitative RT-PCR of β-tubulin isotypes in the control DMSO-treated samples transfected with a scrambled (SCR) siRNA sequence and samples with (50%) silenced ZEB1. The error bars represent the standard deviations of duplicate samples. The data are representative of three independent cell preparations. The statistical significance for Student *t* tests is shown above the bar graphs and level of statistical significance was set at $p = 0.05$.

Because ZEB1 protein persists in both cell lines with 24 h paclitaxel treatment in spite of the decline in ZEB1 mRNA, we wanted to know whether paclitaxel stabilizes ZEB1 protein. We used cycloheximide to reduce protein translation and estimate the half-life of ZEB1 in the presence or absence of paclitaxel. We pretreated cells with 40 nM paclitaxel for 24 h and then replaced the medium with medium containing DMSO (control), 40 nM paclitaxel, 10 μM cycloheximide (to inhibit translation), or 40 nM paclitaxel plus 10 μM cycloheximide for 24 h (Figure 6B). The results show that when protein translation is inhibited, ZEB1 protein stability is not affected by paclitaxel.

We used Western blotting to determine at what time point a measurable loss in ZEB1 and tubulin protein becomes evident with 40 nM paclitaxel treatment (Figure 6C). The half-life of tubulin protein is known to be greater than 24 h.[16] Consistent with this finding, and our estimate that the ZEB1 half-life is greater than 24 h, we did not observe a decrease in either tubulin or ZEB1 protein until 72 h of drug treatment. Flow cytometry data collected after 72 h of drug treatment shows that, similar to the 24 h time point, cells are not blocked in G2/M; however, a larger portion of cells are in the hypodiploid fraction. These data support the findings that ZEB1 and tubulin are coordinately regulated (positive feedback where mRNA levels increase or decrease together). The data also indicate that the increase in miR-200c found after 24 h of treatment with paclitaxel is not directly caused by a decrease in ZEB1 protein. Possible mechanisms are discussed below.

■ DISCUSSION

Although more than two decades have passed since Cleveland and colleagues showed that one or more cellular cofactors are involved in post-transcriptional regulation of β-tubulins,[17−19] little is known about the mechanisms underlying differential regulation of tubulin isotypes. Recently, β-tubulin class III was shown to be associated with survival of ovarian cancer cells,

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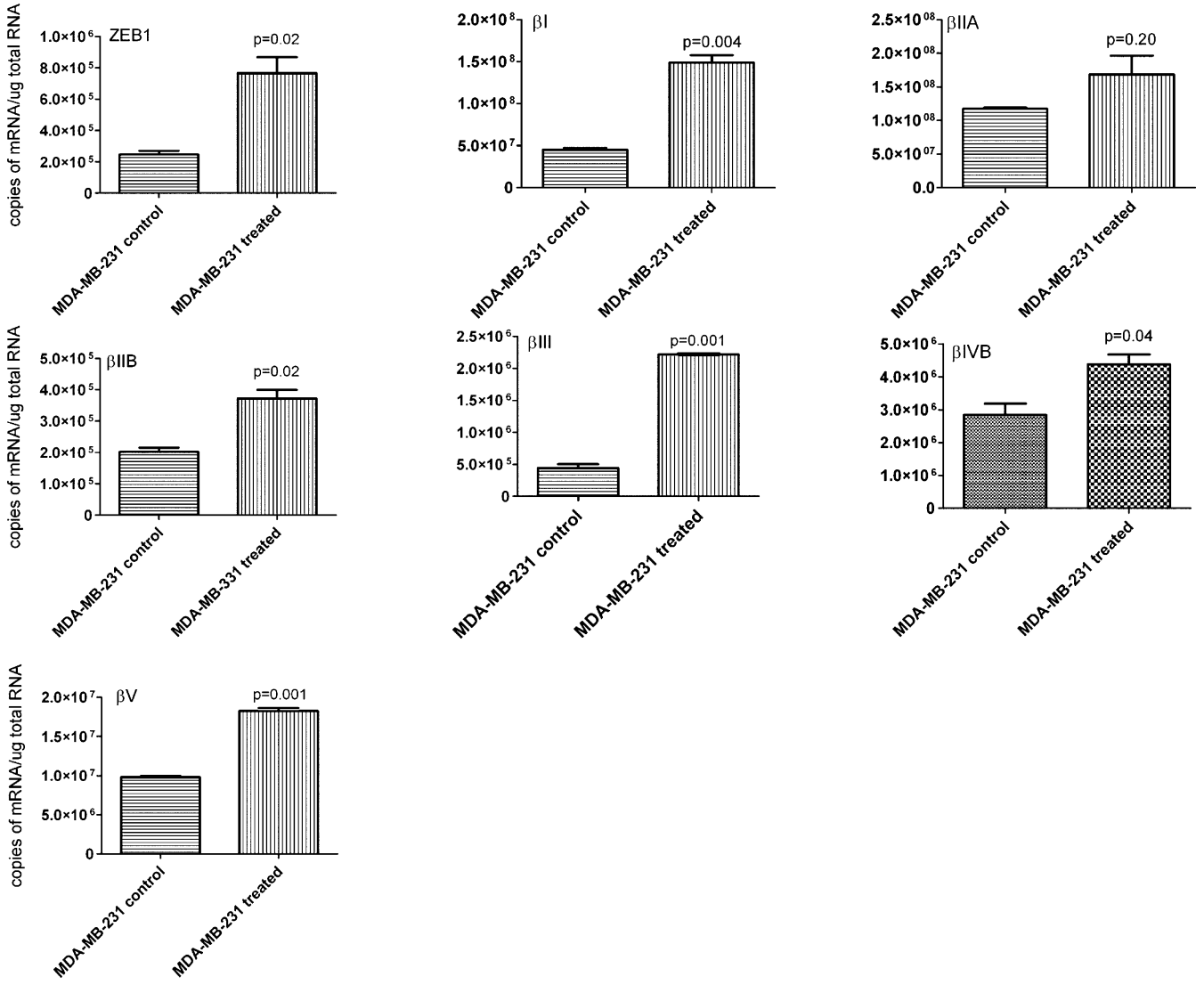

Figure 4. Transfection of MDA-MB-231 cells with ZEB1. Quantitative RT-PCR data were analyzed using standard curves for ZEB1 and each β-tubulin isotype and mRNA copies were normalized to 1 μg of total RNA. Experiments were done with more than three individual cell cultures. A representative experiment is shown. The error bars represent the standard deviation from duplicate samples from a single experiment. The statistical significance for Student t tests is shown above the bar graphs and level of statistical significance was set at $p = 0.05$.

suggesting that mechanisms regulating this isotype also contribute to oncogenesis and possibly metastasis.[5,7,11] Thus, understanding the regulation of β-tubulin isotypes may clarify oncogenic and metastatic pathways. Over the past 15 years, there has been considerable interest in how noncoding micro-RNAs contribute to mRNA and protein regulation. For example, miR-200c was shown to reduce β-tubulin class III in ovarian cell cultures, and restoration of miR-200c to cells and tissues enhanced their sensitivity to paclitaxel.[7] Furthermore, increases in β-tubulin class III and reductions in miR-200c are associated with poor prognosis in ovarian cancer.[5]

In the work reported here, we find in MDA-MB-231 breast cancer cells (known to model the aggressive "triple negative" phenotype) a coordinated regulation involving positive feedback for the mRNA for β-tubulin isotype classes I, III, and IVB and the EMT protein ZEB1, a known target of the tumor suppressor miR-200c. ZEB1 is zinc-finger protein with both repressor and activator functions. It reduces levels of E-cadherin, a marker for cell polarity, leading to cell release and metastasis.[4] Our work demonstrates that ZEB1 and tubulin protein levels parallel mRNA levels after 72 h. Because miR-200c targets ZEB1 mRNA, it is possible that the activity of this tumor suppressor's micro-RNA in part contributes to the positive feedback regulation of ZEB1 and β-tubulin classes I, III, and IVB. We used the prediction software Target Scan (targetscan.org) to identify possible miR-200c binding sites on β-tubulin isotype mRNA. Similar to reports by others,[4,5] we found an exact match for the 8-mer seed sequence on β-tubulin class I and an exact match for the seed sequence 7-mer on β-tubulin class III, supporting the likelihood of coordinated miR-200c regulation of these isotypes. No match for β-tubulin class IVB was found. Preliminary work in our lab, using next generation sequencing, suggests that at least 19 known micro-RNAs are either up-regulated or down-regulated by 24 h, 40 nM paclitaxel treatment of MDA-MB-231 cells. Thus it is likely that micro-RNAs other than miR-200c contribute to the change in the amount of β-tubulin class IVB mRNA observed with drug treatment. β-Tubulin class IIA is also a target for miR-200c;[9] however, we showed previously that miR-100, which also regulates β-tubulin IIA, is downregulated by

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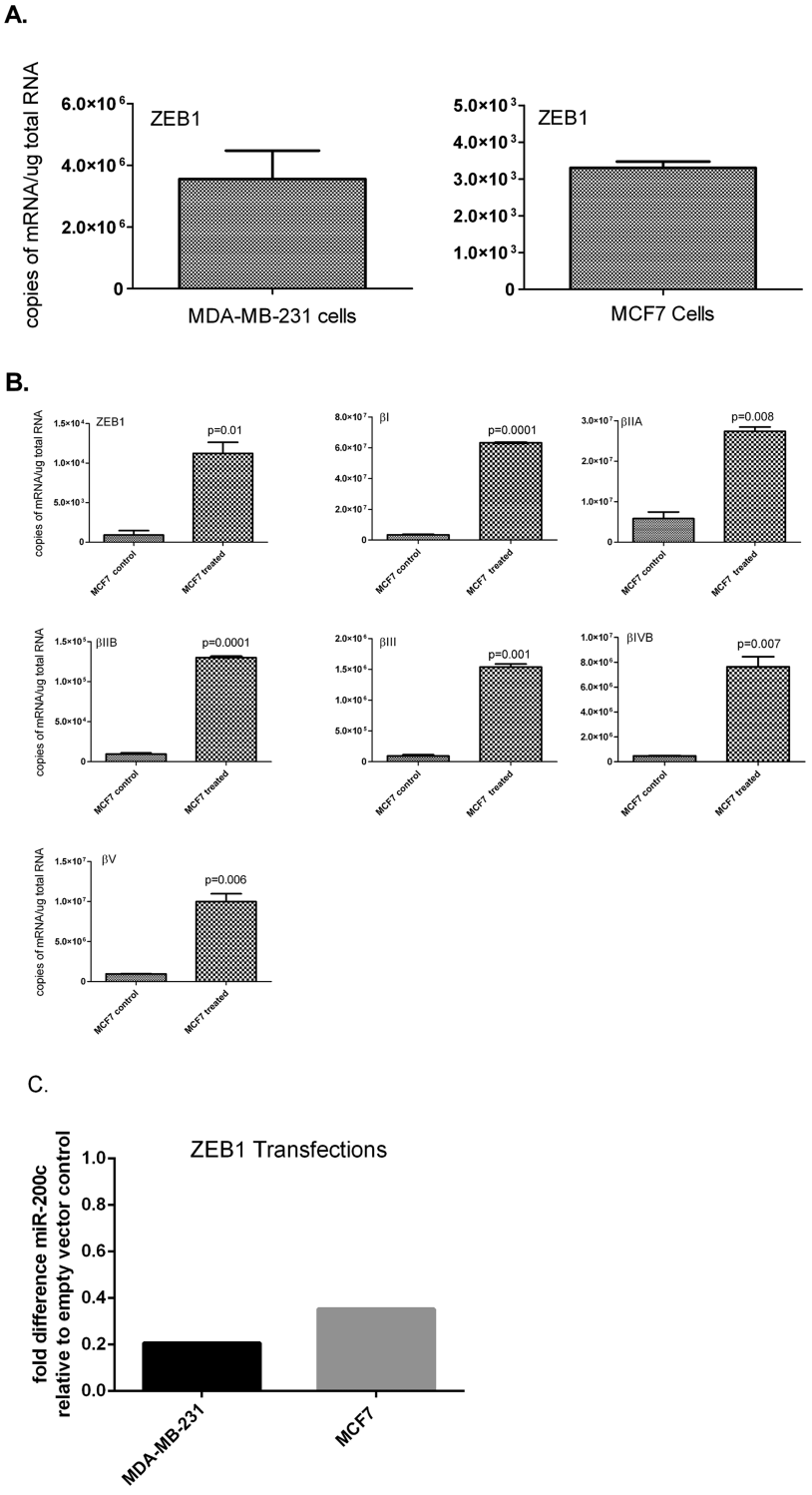

Figure 5. Panel A. qRT-PCR for ZEB1 in MDA-MB-231 cells (left) and MCF7 cells (right). mRNA copies were normalized to 1 μg of total RNA. Nearly 1000-fold fewer copies of ZEB1 mRNA are found in MCF7 cells than in MDA-MB-231 cells. The error bars represent the standard deviation from duplicate independent cell cultures. Panel B. Transfection of MCF7 cells with ZEB1. qRT-PCR data were analyzed using standard curves for ZEB1 and each β-tubulin isotype, and mRNA copies were normalized to 1 μg of total RNA. Experiments were done with more than three individual cell cultures. A representative experiment is shown. The error bars represent the standard deviation from duplicate samples from a single experiment. The statistical significance for Student *t* tests is shown above the bar graphs, and level of statistical significance was set at $p = 0.05$. Panel C. qRT-PCR data from ZEB1 transient tranfections from panels A and B were analyzed as the fold difference in miR-200c relative to the empty vector control.

paclitaxel treatment.[14] Thus, no change or an increase in β-tubulin IIA is not surprising.

Because ZEB1 is a known inducer of EMT in cancer cells, our work suggests that associated changes in β-tubulin isotype levels could be used as biomarkers for metastatic disease. β-Tubulin class III protein is in low abundance or absent from normal tissues, and because of this, an increase is readily measurable by Western blotting or immunohistochemistry. Changes in the

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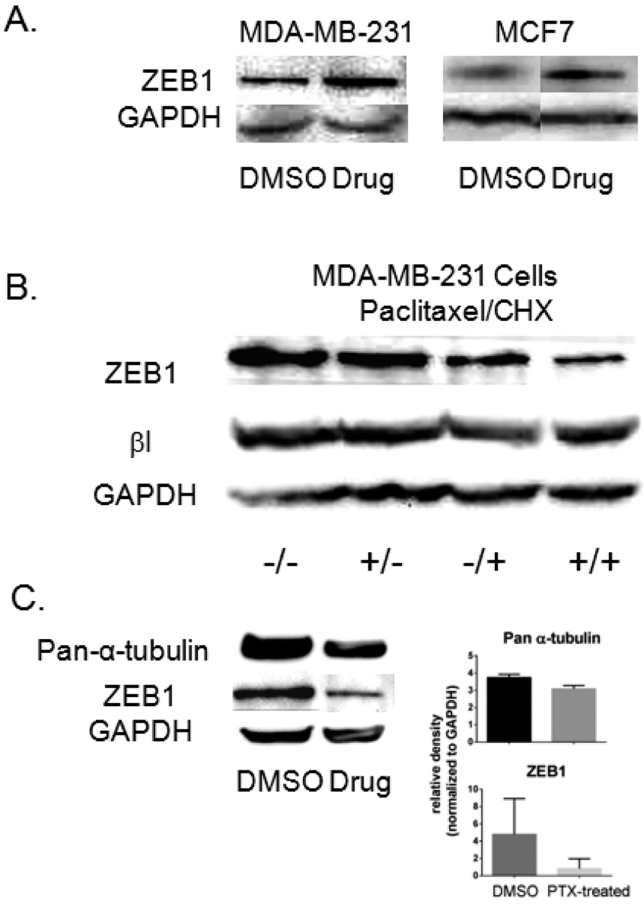

Figure 6. Western blots of paclitaxel-treated cells. Panel A. MDA-MB-231 and MCF7 cells were treated with either 40 nM paclitaxel or the same volume of DMSO for 24 h. Blots were reacted with ZEB1 antibody or GAPDH as the loading control. Panel B. Cells were pretreated with 40 nM paclitaxel for 24 h, and then the medium was removed and replaced with medium containing the following: lane 1, DMSO (−/−); lane 2, 40 nM paclitaxel (+/−); lane 3, 10 μM cycloheximide (CHX) (−/+); lane 4, 40 nM paclitaxel plus 10 μM CHX (+/+). Western blots were reacted with monoclonal antibodies raised against ZEB1, β-tubulin class I, or GAPDH as the loading control. The amount of ZEB1 protein is shown to decrease when translation is inhibited by CHX, indicating that the protein is not stabilized by paclitaxel. Panel C. MDA-MB-231 cells were treated with DMSO or 40 nM paclitaxel (PTX) for 72 h. Left panel: Western blots were reacted with pan-α-tubulin antibody, DMIA, ZEB1 antibody, or GAPDH antibody as the loading control. Right panel: Relative density quantitation of Western blots normalized to GAPDH. Error bars represent the standard deviation of mean densities from two individual DMSO and paclitaxel (PTX)-treated cell cultures preparations.

more abundant β-tubulin isotypes such as class I or IVB are more difficult to detect because the signal is constitutively present in many human tissues.[20] We propose that changes in at least β-tubulin class I, III, and IVB all occur as cells in tissues become dysregulated and potentially metastatic; however, changes in β-tubulin class III are most likely to be measurable relative to other β-tubulin classes. Further, an increase in β-tubulin class III is associated with diminishing amounts of the tumor suppressor micro-RNA, miR-200c.

Our work demonstrates that, in MDA-MB-231 breast cancer cells, the decline in β-tubulin isotypes I, III, and IVB in response to paclitaxel is associated with an increase in miR-200c. This increase likely contributes to the reduction in β-tubulin isotypes levels. However, as noted above, drug treatment alters levels of several micro-RNAs that could contribute to the reduction in tubulin mRNA. Our results raise the possibility that the effectiveness of paclitaxel may be in part due to an increase in the tumor suppressor miR-200c. We showed that ZEB1 protein levels do not decrease significantly until 72 h of 40 nM paclitaxel treatment. Thus, the reason for the increase in miR-200c is not due to a decrease in total ZEB1 protein. Possible mechanisms to explain the increase in miR-200c with paclitaxel treatment could involve sequestration of ZEB1 and/or other signaling proteins in the cytoplasm due to alterations in microtubule functions. For example, recently it was reported that ZEB1 and androgen receptors regulate each other in MDA-MB-231 cells through positive feedback.[15] Also, Giannakakou and colleagues showed that paclitaxel treatment can sequester androgen receptors as well as other proteins that track along microtubules in the cytoplasm.[22] Thus, paclitaxel treatment could sequester ZEB1 or other proteins that regulate miR-200c in the cytoplasm, resulting in an increase in miR-200c due to their reciprocal relationship. Investigations of ZEB1 intracellular transport will help clarify the mechanisms underlying our observations that the regulation of ZEB1 and β-tubulin classes I, III, and IVB mRNAs are directly or indirectly coordinated.

Different from MDA-MB-231 cells, MCF7 cells are known to have low levels of ZEB1 and relatively high levels of miR-200c.[4] We find that, in fact, there is at least a 1000-fold difference in ZEB1 mRNA copy numbers in these two cell lines. This difference suggests that changes in β-tubulin isotypes in response to paclitaxel would be different in the two cell lines. Indeed, we showed that high doses of paclitaxel (400 nM) induce large increases in β-tubulin classes IIA, III, and V in MCF7 cells,[14] which was quite different from the changes we observed in MDA-MB-231 cells. More recently, we found significant increases in the β-tubulin isotypes with lower doses of paclitaxel in MCF7 cells (40 nM data not shown). Further, our data suggest that β-tubulin classes IIA and V are not regulated by miR-200c, and our recent work suggests these isotypes are, in part, regulated by miR-100.[14] Cheng and colleagues showed that differential expression of key micro-RNA processing genes (Ago2 and Dicer) in estrogen receptor positive (MCF7) and negative (MDA-MB-231) cell lines may explain differential regulatory effects of micro-RNAs in breast cancer cells.[21] These data together indicate that paclitaxel treatment may be associated with increased or decreased activity of miR-200c depending upon the cell phenotype and that drug resistance may, in part, depend upon the response of miR-200c.

Our work has implications for β-tubulin class III as a "survival factor" in concert with GTPases that promote tumor cell survival.[11] The results presented here suggest that up-regulation of β-tubulin class III in specific cell types is also closely linked to EMT induced by ZEB1 and could indicate an aggressive tumor phenotype. Tumor cells that fail to respond to paclitaxel by reducing β-tubulin class III mRNA may in fact be drug resistant. This study supports the work of others indicating that the persistence of β-tubulin class III protein may be a biomarker for paclitaxel resistance.[7]

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Notes

The authors declare no competing financial interest.

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