MicroRNA-31 Sensitizes Human Breast Cells to Apoptosis by Direct Targeting of Protein Kinase C ε (PKCe)*

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Background: The role of miR-31 in sensitivity of breast tumors to anti-cancer treatment is unknown.

Results: miR-31 directly targets PRKCE and thereby suppresses NF-κB activity and induces apoptosis and sensitivity to anti-cancer treatments via down-regulation of BCL2 expression.

Conclusion: miR-31 indirectly down-regulates BCL2 expression.

Significance: This study adds an important aspect to the tumor-suppressive potential of miR-31.

MicroRNAs post-transcriptionally regulate gene expression and thereby contribute to the modulation of numerous complex and disease-relevant cellular phenotypes, including cell proliferation, cell motility, apoptosis, and stress response. In breast cancer cell systems, miR-31 has been shown to inhibit cell migration, invasion, and metastasis. Here, we link enhanced expression of miR-31 to the inhibition of the oncopgenic NF-κB pathway, thus supporting the tumor-suppressive function of this microRNA. We identified protein kinase C epsilon (PKCe encoded by the PRKCE gene) as a novel direct target of miR-31 and show that down-regulation of PKCe results in impaired NF-κB signaling, enhanced apoptosis, and increased sensitivity of MCF10A breast epithelial and MDA-MB-231 triple-negative breast cancer cells toward ionizing radiation as well as treatment with chemotherapeutics. Mechanistically, we attribute this sensitization to anti-cancer treatments to the PRKCE-mediated down-regulation of the anti-apoptotic factor BCL2. In clinical breast cancer samples, high BCL2 expression was associated with poor prognosis. Furthermore, we found an inverse correlation between miR-31 and BCL2 expression, highlighting the functional relevance of the indirect down-regulation of BCL2 via direct targeting of PRKCE by miR-31.

In women, breast cancer is the most common cancer type and the second leading cause of cancer mortality in females worldwide (1). Breast cancer can be categorized into different subtypes based on the expression of certain protein markers. Tumors expressing estrogen receptor (ER)3 and often also progesterone receptor are classified as ER-positive (ER+) tumors, whereas tumors with genomic amplification and high surface expression of the human epidermal receptor 2 (HER2) are classified as HER2-positive (HER2+). These tumors are commonly treated by targeted therapies. Tumors that are negative for ER, progesterone receptor, and HER2 are referred to as triple-negative or basal-like tumors (2). Due to the lack of cancer-specific, targeted treatment options, triple-negative breast cancers are the most aggressive breast tumors and are associated with poor prognosis (3). One major reason for cancer-associated deaths is the development of resistance. The molecular mechanisms leading to therapy resistance are diverse and incompletely understood. However, it is well accepted that the deregulation of apoptosis-associated genes, as well as aberrant survival signals, such as constitutively active nuclear factor κB (NF-κB) signaling, constitute important resistance mechanisms (4, 5).

The NF-κB transcription factor transcriptionally triggers expression of genes controlling tumor growth, invasion, and cellular survival, thus having a central role in tumor initiation, tumor progression, and the response to anti-cancer treatments (4). Regulated genes include the cell cycle regulator cyclin D1 (CCND1) or the anti-apoptotic factor B-cell/CLL leukemia 2 (BCL2) (6, 7). NF-κB signaling has been reported to be constitutively active in >80% of all breast tumors and its inhibition has been demonstrated to induce apoptosis in vitro and in vivo and to thereby reduce tumor growth (8, 9). Furthermore, inhibition of NF-κB has been linked to reduced invasiveness and metastasis formation (10).

miRNAs are endogenous small non-protein-coding RNAs of ~22 nucleotides in length. They constitute a large class of mostly negative regulators of gene expression and mainly act by direct base pairing with a partially complementary target site within the 3′-untranslated region (3′-UTR) of their target messenger RNAs (mRNAs). Identification of putative targets of a miRNA is often performed using bioinformatic algorithms that predict potential miRNA binding sites within 3′-UTRs, mainly based on perfect matching of the miRNA seed region (nucleotides 2–8) to the target site in conjunction with evolutionary conservation of the respective target site (11). miRNAs regulate a vast range of cellular processes including proliferation, cell motility, differentiation, and apoptosis. Furthermore, miRNA expression has been found to be altered in various human cancer entities, including breast cancer (12). For instance, we have previously shown that miR-520c and miR-200c inhibit meta-

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3 The abbreviations used are: ER, estrogen receptor; miRNA, microRNA; qRT-PCR, quantitative RT-PCR.
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static cell invasion and linked low miR-520c expression in ER− breast tumors to an increased risk of lymph node metastases (13, 14). In contrast, other miRNAs such as miR-21 have been described to be up-regulated in breast tumors compared with normal breast tissue and to promote carcinogenesis by down-regulating several tumor suppressor genes (15, 16). These findings highlight the ability of miRNAs to modulate cancer initiation and progression by regulating critical cancer-related genes and signaling pathways.

miR-31 is a pleiotropically acting miRNA implicated in cancer as it has been shown to inhibit invasion and metastasis of breast cancer and other cancer types, including ovarian cancer or leukemia (17–19). Its expression inversely correlates with metastasis in human breast cancer patients and overexpression of this miRNA suppresses metastasis in vitro. It has been demonstrated to suppress a number of metastasis-promoting target genes thereby affecting several steps of metastasis formation such as local invasion, extravasation, and metastatic colonization (18, 20, 21). Thus, deregulation of this miRNA interferes with multiple steps of the invasion-metastasis cascade. Furthermore, miR-31 has been described to target the NF-κB-inducing kinase (MAP3K14) leading to impaired NF-κB-mediated transcription and thereby to increased apoptosis in T-cells (19). However, miR-31 has not been linked to apoptosis and resistance to chemo- and radiotherapy in breast cancer cells.

Here, we show that miR-31 inhibits TNF-α induced NF-κB signaling in breast cells. We identified protein kinase C ε (PRKCE) to be a novel direct target of miR-31, accounting for the observed reduction of NF-κB activity. Furthermore, we show that overexpression of miR-31 leads to increased sensitivity of breast cells toward chemotherapeutic and radiotherapy and that this is mediated by direct targeting of PRKCE. Mechanistically, we attribute these phenotypes to the indirect PRKCE-mediated down-regulation of BCL2 expression, in response to overexpression of miR-31 (22) because stable ectopic expression of both PRKCE and BCL2 abrogate the sensitizing effect of miR-31 in MCF10A cells. In addition, we show an inverse correlation of BCL2 and miR-31 expression in breast cancer specimens. These findings suggest that miR-31 does not only affect metastatic spread, but also tumor growth and therapeutic resistance, thereby further highlighting the tumor-suppressive potential of this miRNA in breast cancer.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—MDA-MB-231, MCF-7, and MCF10A cell lines were obtained from ATCC (Manassas, VA) in 2009 and 2010. HEK293FT cells were obtained from Invitrogen in 2009. Cell lines were verified using the cell line authentication service at the DFKZ Core Facility by Multiplex human cell authentication in 2011 (23). Human recombinant TNF-α (Sigma-Aldrich) was used at a final concentration of 20 ng/ml. Cells were maintained as described previously (13). All cell culture media and reagents were from Invitrogen. MCF10A cells stably overexpressing ORFs were generated by retroviral transduction using the pBABE vector system.

Transfections—All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. siRNAs targeting PRKCE and BCL2 were purchased from Ambion (Invitrogen). siAllStars siRNA (Qiagen, Hilden, Germany) was used as a non-targeting control (siCtrl). miRIDIAN miRNA mimics and negative controls were obtained from Dharmacon (Lafayette, CO). siRNAs and miRNA mimics were used at a final concentration of 30 nM.

Luciferase Reporter Assays—Luciferase reporter assays have been performed as described previously (13).

RNA Isolation and Real-Time PCR—Total RNA and microRNA were isolated from cells using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was carried out with the Revert Aid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). The qRT-PCR reactions for target genes were performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Weiterstadt, Germany), using probes from the Universal Probe Library (Roche Diagnostics). The housekeeping genes HPRT1 and GAPDH were used for normalization of mRNA analysis.

Antibodies and Western Immunoblotting—Western immunoblotting was performed as described previously (13). The following antibodies were used: rabbit monoclonal phospho-NF-κB p65 (Ser-536), rabbit anti-PKCε (22B10) (both Cell Signaling Technology, Danvers, MA), rabbit anti-actin (20-33) (Sigma-Aldrich). Blots were probed with IRDye® 680 or IRDye® 800 conjugated antibodies (H+L) (LI-COR, Lincoln, NE), and bands were visualized using an Odyssey scanner (LI-COR). Primary antibodies were used at a 1:1000 dilution and secondary antibodies were used at a 1:10,000 dilution.

NF-κB Translocation Assay—NF-κB translocation assays were performed as described previously (13) using rabbit polyclonal NF-κB p65 primary antibody (A) (Santa Cruz Biotechnology, Santa Cruz, CA). NF-κB signaling was triggered by stimulation with TNF-α for 15 min. Images for quantitative analysis were acquired using an Olympus ScanR high-content screening microscope (Olympus, Hamburg, Germany), and cells with mainly nuclear or mainly cytoplasmic localization of p65 were quantified automatically using the built-in ScanR analysis software application.

Apoptosis Assay—Cells were transfected in black 96-well glass-bottomed plates (GE MatriCal, Spokane, Washington), and 48 h after transfection, cells were treated with 0.3 μl of NucView-488 per well for 30 min. Thereafter, cells were washed with PBS and fixed with ice-cold methanol, and nuclei were stained using DAPI (Sigma-Aldrich). Images were acquired using an Olympus ScanR high-content screening microscope (Olympus, Hamburg, Germany) and nuclei as well as cells positive for NucView-488 staining were automatically counted using the ScanR analysis software.

Cell Viability Assays—72 h after γ-irradiation or treatment with staurosporine (Roche Applied Science) or doxorubicin (Sigma-Aldrich), cell viability was assessed using CellTiter Glo (Promega, Madison, WI) according to the manufacturer’s recommendations. IC50 concentrations were determined using GraphPad Prism.

Statistical Analyses—Data are presented as mean ± S.D. Samples were analyzed by two-tailed unpaired Student’s t test, unless otherwise mentioned, and p values < 0.05 were considered as being statistically significant. p values < 0.05, < 0.01,
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Overexpression of miR-31 reduces NF-κB activity by a mechanism independent from the known direct targets RhoA and NF-κB-inducing kinase (NIK, MAP3K14). a, NF-κB activity in HEK293FT cells was analyzed by an NF-κB reporter luciferase assay. Cells were co-transfected with the 3xKBL luciferase reporter plasmid, the pMir-Report-β-Gal reporter plasmid for normalization and the indicated MIRIDIAN miRNA mimics. 48 h after transfection, cells were stimulated using 20 ng/ml TNF-α for 5 h, and relative luciferase activity was quantified (RLU, relative luciferase units). b, NF-κB activity in HEK293FT cells was analyzed by Western blot staining for active phosphorylated p65 (Ser-536). Cells were transfected with the indicated miRNA mimics. 48 h after transfection, cells were stimulated using 20 ng/ml TNF-α for 15 min, and protein lysates were prepared. GAPDH was used as loading control (ctr). c, NF-κB activity in MCF10A (c) and MDA-MB-231 (d) cells was analyzed by Western blot using the indicated miRNA mimics. 48 h after transfection, cells were stimulated using 20 ng/ml TNF-α for 15 min and immunostained for p65. Nuclei were stained with DAPI, and the ratio of “active” cells with mainly nuclear localization of p65 over “passive” cells with mainly cytoplasmic localization was determined using an Olympus ScanR high-content screening microscope. d, expression levels of miR-31 upon transfection with miRNA mimics were determined by qRT-PCR. e, NF-κB activity in HEK293FT cells was analyzed by NF-κB reporter luciferase assay. Cells were co-transfected with the 3xKBL luciferase reporter plasmid, the pMir-Report-β-Gal reporter plasmid for normalization and the indicated siRNAs. 48 h after transfection, cells were stimulated using 20 ng/ml TNF-α for 5 h, and relative luciferase activity was quantified.

and < 0.001 are indicated with one, two, and three asterisks, respectively. Data obtained from breast cancer patient samples were analyzed using GraphPad Prism. Expression of miR-31 and BCL2 mRNA was correlated using Pearson’s correlation. Survival data were analyzed using log-rank (Mantel-Cox) test.

RESULTS

Overexpression of miR-31 Reduces NF-κB Activity—In a genome-wide miRNA screen for miRNA regulators of TNF-α-induced NF-κB signaling, we identified the tumor suppressor miRNA, miR-31, to down-regulate NF-κB-driven transcription. For the initial screen, miRNA mimics were co-transfected into HEK293FT cells with a reporter plasmid containing three consensus NF-κB binding sites followed by a luciferase gene. NF-κB signaling was triggered 48 h later by stimulating the cells with the proinflammatory cytokine TNF-α. Stimulation with TNF-α strongly induced the activity of the NF-κB reporter. However, this induction was significantly reduced by overexpression of miR-31 (Fig. 1a). Alternative miRNAs with seed region similarity to miR-31 upon transfection with miRNA mimics were determined by qRT-PCR. f, NF-κB activity in HEK293FT cells was analyzed by NF-κB reporter luciferase assay. Cells were co-transfected with the 3xKBL luciferase reporter plasmid, the pMir-Report-β-Gal reporter plasmid for normalization and the indicated siRNAs. 48 h after transfection, cells were stimulated using 20 ng/ml TNF-α for 5 h, and relative luciferase activity was quantified.
NF-κB signaling has been reported to be constitutively active in a large fraction of breast tumors, and its inhibition has been shown to interfere with tumor growth as well as invasiveness and apoptosis of breast cancer cells (8–10). Consequently, we asked whether ectopic overexpression of miR-31 might also affect NF-κB activity in cultured cells derived from breast tissue. Because nuclear translocation of p65 is indicative of the activity of the canonical NF-κB pathway, we transfected non-tumorigenic immortalized breast epithelial MCF10A cells as well as aggressive triple-negative MDA-MB-231 breast cancer cells with mimic control or mimic miR-31 and analyzed NF-κB activity in response to TNF-α stimulation by applying a microscopic p65 nuclear translocation assay. Depending on the ratio of nuclear versus cytoplasmic intensity, cells were classified as either active cells with mainly nuclear localization of p65 or passive cells with mainly cytoplasmic staining. Stimulation of both cell lines with TNF-α resulted in a substantial increase in the ratio of active compared with passive cells in mimic control transfected samples. However, overexpression of miR-31 strongly reduced the TNF-α-induced effect on NF-κB translocation in both cell lines (Fig. 1, c and d; expression levels of miR-31 are depicted in Fig. 1e). These results demonstrate that miR-31 is a negative regulator of TNF-induced NF-κB activation in cells derived from breast tissue. This inhibition did not translate into inhibition of expression of cytokines regulated by NF-κB (e.g. IL-6 or IL-8).

Reduced NF-κB Activity in Breast Cell Lines Is Independent of Targeting NF-κB-inducing Kinase and RhoA—Next, we aimed to link the reduced activity of NF-κB to the down-regulation of direct targets of miR-31. Two genes implicated in NF-κB signaling are among the published validated targets of miR-31: the NF-κB-inducing kinase (MAP3K14) and the RhoA GTPase (18, 19). Both direct targets act as activators of NF-κB signaling (25, 26). Hence, their down-regulation by miR-31 might mediate the reduction of NF-κB signaling in response to miR-31 overexpression. To test this hypothesis, we analyzed the effect of knockdown of MAP3K14 and RhoA on the activity of the NF-κB reporter plasmid in HEK293FT cells stimulated with TNF-α (Fig. 1f). Although knockdown of MAP3K14 did not induce a significant effect on NF-κB activity when compared with non-targeting control siRNA, knockdown of RhoA indeed reduced NF-κB activity. However, the inhibitory effect induced by the siRNA targeting RhoA was substantially weaker compared with the effect of miR-31 overexpression as shown in Fig. 1a, suggesting an additional direct target of miR-31 was responsible for the inhibition of NF-κB signaling.

**PRKCE Is a Direct Target of miR-31**—To identify further direct targets of miR-31 within the NF-κB pathway, we performed bioinformatic target prediction analysis using TargetScan (version 6.2) (27). The top 20 predictions according to their context+ score along with their known functional associations with NF-κB activity are listed in Table 1. Protein kinase C ε (PRKCE) ranked among the top 10 predictions according to their context+ score and was the only gene known to be involved in regulation of TNF-α-induced NF-κB signaling (28). The 3′-UTR of this kinase contains one conserved and two poorly conserved predicted target sites for miR-31. A scheme of the putative miR-31 target sites as well as of the luciferase reporter plasmids used to validate direct targeting is depicted in Fig. 2a. We first analyzed the effects of miR-31 overexpression on mRNA levels of PRKCE both in MCF10A and MDA-MB-231 cells; however, mRNA expression of PRKCE was unchanged (Fig. 2b). Because direct targeting by a miRNA does not necessarily lead to mRNA degradation, we next analyzed

**TABLE 1**

**Predicted targets of miR-31 ranked according to their context+ scores**

| Target gene | Representative transcript | Gene name | Conserved sites | Poorly conserved sites | Total context + score | Known activation of NF-κB |
|-------------|---------------------------|-----------|----------------|-----------------------|----------------------|--------------------------|
| RSN1        | NM_018364                 | Round spermatid basic protein 1 | 2              | 3                     | −0.97                | None                     |
| ARHGEF2     | NM_001162383              | Rho/Rac guanine nucleotide exchange factor (GEF) 2 | 1              | 2                     | −0.74                | LPS-induced              |
| JDE         | NM_01165946               | Insulin-degrading enzyme 3 | 2              | 1                     | −0.69                | None                     |
| NR5A2       | NM_003822                 | Nuclear receptor subfamily 5, group A, member 2 | 2              | 1                     | −0.67                | None                     |
| SH2D1A      | NM_001114937              | SH2 domain containing 1A | 2              | 0                     | −0.65                | None                     |
| ZNF512      | NM_032434                 | Zinc finger protein 512 | 2              | 1                     | −0.65                | None                     |
| PRKCE       | NM_003400                 | Protein kinase C ε | 1              | 2                     | −0.64                | TNF-α-induced            |
| PKR3CA      | NM_002645                 | Phosphoinositide-3-kinase, class 2, α polypeptide | 2              | 0                     | −0.59                | None                     |
| PEX5        | NM_000319                 | Peroxisomal biogenesis factor 5 | 1              | 1                     | −0.56                | None                     |
| SATB2       | NM_00175209               | SATB homeobox 2 | 2              | 0                     | −0.56                | None                     |
| AKAP7       | NM_004842                 | A kinase (PRKA) anchor protein 7 | 2              | 1                     | −0.55                | None                     |
| TSGA10      | NM_025244                 | Testis-specific, 10 | 1              | 0                     | −0.54                | None                     |
| RHOBTB1     | NM_001242359              | Rho-related BTB domain containing 1 | 1              | 1                     | −0.54                | None                     |
| OAS2        | NM_016817                 | 2′,5′-Oligoadenylate synthetase 2, 69/71kDa | 1              | 2                     | −0.53                | None                     |
| SEPH51      | NM_00195602               | Selenophosphate synthetase 1 | 1              | 1                     | −0.51                | None                     |
| DJUSP7      | NM_001947                 | Dual specificity phosphatase 7 | 1              | 1                     | −0.5                | None                     |
| PPP1R9A     | NM_00116610               | Protein phosphatase 1, regulatory (inhibitor) subunit 9A | 1              | 0                     | −0.5                | None                     |
| RNF144B     | NM_182757                 | Ring finger protein 144B | 1              | 1                     | −0.48                | None                     |
| KANK1       | NM_015158                 | KN motif and ankyrin repeat domains 1 | 1              | 0                     | −0.48                | None                     |
| SLC1A2      | NM_001195728              | Solute carrier family 1, member 2 | 2              | 2                     | −0.48                | None                     |
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PKCe protein expression in MCF10A cells upon overexpression of miR-31 (Fig. 2c) and indeed observed a strong down-regulation of PKCe protein expression as compared with control cells.

To prove that down-regulation of PKCe protein level is indeed caused by a direct interaction of miR-31 with the 3'-UTR of PRKCE mRNA, we performed luciferase reporter assays. The wild-type full-length 3'-UTR of PRKCE as well as UTRs mutated either in a single miR-31 target site or in all three putative target sites for miR-31 were cloned into a luciferase reporter construct. Upon transfection of the wild-type (WT) construct into MCF-7 cells, overexpression of miR-31 reduced the relative activity of Renilla luciferase by 50% compared with the negative control. Mutation of either one of the putative...
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Down-regulation of PRKCE Accounts for Impaired Activity of NF-κB Caused by miR-31—Having validated PRKCE as a direct target of miR-31, we next aimed to establish a connection between this novel target gene and NF-κB activity. For that purpose, we first analyzed the effect of PRKCE knockdown in the NF-κB reporter luciferase assay in HEK293FT cells (Fig. 3a). Indeed, knockdown of PRKCE reduced the NF-κB activity at an extent that was similar to overexpression of miR-31 (Fig. 1a), thus indicating that down-regulation of PRKCE might indeed account for the reduced transcriptional activity of NF-κB upon overexpression of miR-31. To further investigate this, we analyzed the effect of PRKCE knockdown on the nuclear translocation of p65 in MCF10A (Fig. 3b) and MDA-MB-231 cells (Fig. 3c). In both breast cell lines, reduced expression of PRKCE resulted in a significant inhibition of nuclear translocation of p65 indicative of reduced transcriptional activity of NF-κB. Hence, knockdown of PRKCE phenocopied the effect of miR-31 overexpression on p65 localization in MCF10A and MDA-MB-231 cells (Fig. 1, c and d). Reduced activity of NF-κB signaling in response to knockdown of PRKCE is further endorsed by reduced levels of active p65 as measured by Western Immunoblotting for p65 phosphorylated at serine residue Ser-536 (Fig. 3d). In summary, our results demonstrate that down-regulation of PRKCE partially accounts for the reduced activity of NF-κB in cells overexpressing miR-31, independent of the cellular context.

miR-31 Overexpression as Well as Down-regulation of PRKCE Result in Apoptosis and Enhanced Chemo- and Radiosensitivity—Because impaired activity of NF-κB has been shown to be associated with increased cellular sensitivity to apoptosis (8, 9), we next analyzed whether overexpression of miR-31 and knockdown of PRKCE would affect apoptosis in MCF10A and MDA-MB-231 cells. Indeed, both enhanced expression of miR-31 (Fig. 4a) as well as impaired expression of PRKCE (Fig. 4b) induced a >2-fold increase in basal apoptosis levels in the two cell lines. This was in line with our observation that both conditions were also associated with mildly impaired cell proliferation in these cell lines.

With respect to the clinical outcome, not only cell proliferation and apoptosis, but rather resistance of breast cancer cells to chemotherapy and irradiation are of importance. As NF-κB signaling has been implicated in these phenotypes, we hypothesized that overexpression of miR-31 might also impact the sensitivity of cells to treatment with apoptosis-inducing agents as well as ionizing radiation via down-regulation of PRKCE. To test this hypothesis, MCF10A and MDA-MB-231 cells were transfected with mimic miR-31 or siPRKCE along with the respective negative controls and irradiated with 5 Gray (Gy) 24 h after transfection. Next, cells were grown for an additional 72 h, and viability was monitored. To visualize the cytotoxic effect of irradiation, independent of changes in cell proliferation induced by overexpression of miR-31 or knockdown of PRKCE, the ratio of the normalized viability of irradiated over non-irradiated cells was computed. Indeed, both the enhanced expression of miR-31 (Fig. 4c) and decreased expression of PRKCE (Fig. 4d) significantly reduced the viability of MCF10A as well as MDA-MB-231 cells in response to ionizing radiation.

Similarly, MCF10A cells transfected with mimic miR-31 or siPRKCE were treated with different concentrations of the apoptosis inducer staurosporine 24 h after transfection and allowed to grow for 72 h before viability was assessed. Similar to the results obtained upon irradiation, both overexpression of miR-31 (Fig. 4e) and knockdown of PRKCE (Fig. 4f) sensitized MCF10A cells to treatment with staurosporine. Cells transfected with mimic control exhibited an IC_{50} concentration of 6.0 nM ± 1.3 nM, whereas cells transfected with mimic miR-31 were significantly more sensitive to staurosporine treatment showing the half-maximal effect at a concentration of only 2.3
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FIGURE 4. miR-31 sensitizes MCF10A and MDA-MB-231 cells to apoptosis, to ionizing radiation, and to cytotoxic compounds by reducing PRKCE expression. Apoptosis in MCF10A and MDA-MB-231 cells transfected with miRNA mimics (a) and siRNAs was assessed by the NucView-488 assay (b). Cells were transfected with the indicated miRNA mimics or siRNAs. 48 h after transfection, apoptotic cells were stained with NucView-488, and nuclei of all cells were stained with DAPI. Plates were acquired using an Olympus ScanR high-content screening microscope, and nuclei as well as cells positive for NucView-488 staining were automatically counted using the ScanR analysis software. Radiosensitivity of MCF10A and MDA-MB-231 cells transfected with miRNA mimics (c) and siRNAs (d) was determined by Celltiter Glo cell viability assay 72 h after irradiation. The ratio of the viability of cells irradiated with 5 Gray over the viability of non-irradiated cells is plotted. Sensitivity of MCF10A cells transfected with miRNA mimics (e) and siRNAs (f) to staurosporine was determined by Celltiter Glo cell viability assay 72 h after treatment. Sensitivities are plotted on log-10 scale, and IC50 values are listed in the table below. Sensitivity of MCF10A (g) and MDA-MB-231 (h) cells transfected with miRNA mimics or siRNAs to the intercalating chemotherapeutic drug doxorubicin was determined by Celltiter Glo cell viability assay 72 h after treatment. Concentrations are plotted on log-10 scale. i, breast and ovarian cancer cell lines contained in the NCI-60 cell line panel were used to correlate miR-31 expression with doxorubicin sensitivity. Ctrl, control.

Sensitivity of MCF10A cells to staurosporine (Figures 4e and f, IC50 in nM)

|                | mimic-ctrl | mimic-miR-31 | p-value |
|----------------|------------|--------------|---------|
| miR-ctrl       | 6.0 +/- 1.3| 2.3 +/- 0.5  | <0.01   |
| siCtrl         | 9.0 +/- 2.1| 4.1 +/- 1.1  | <0.01   |

|                | mimic-ctrl | mimic-miR-31 | siPRKCE |
|----------------|------------|--------------|---------|
| miR-ctrl       |            |              |         |
| siCtrl         |            |              |         |
| siPRKCE        |            |              |         |
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miR-31 overexpression induces apoptosis and enhanced sensitivity to chemotherapeutic drug doxorubicin in MCF10A and MDA-MB-231 cells. This effect is at least partially mediated by down-regulation of PRKCE due to direct targeting of miR-31.

Apoptosis as well as Chemo- and Radioresensitivity Are Caused by Impaired Expression of BCL2—Analyzing gene expression profiling data of MDA-MB-231 cells overexpressing miR-31, we identified BCL2, a potent anti-apoptotic gene, to be down-regulated upon overexpression of miR-31. Of note, none of the other well-known anti-apoptotic genes controlled by NF-κB such as FLIP or XIAP were significantly down-regulated in this experiment, suggesting an alternative regulation of BCL2 expression in this system. As PRKCE has not only been reported to contribute to the activation of NF-κB signaling but also to control the expression of the anti-apoptotic BCL2 gene by activating the transcription factor CREB (22), we hypothesized that down-regulation of PRKCE by overexpression of miR-31 might also result in reduced expression of BCL2. This link could serve as a further explanation for the apoptosis inducing phenotype of enhanced miR-31 expression that we had observed in MCF10A and MDA-MB-231 cells. Indeed, BCL2 expression was strongly reduced in the two cell lines in response to down-regulation of PRKCE either indirectly by overexpression of miR-31 or by direct siRNA-mediated knockdown of PRKCE (Fig. 5a, corresponding protein expression as determined by Western blot is depicted below). However, down-regulation of BCL2 is not caused by a direct interaction between miR-31 and the 3'-UTR of BCL2 because we did not observe any effect in a 3'-UTR reporter luciferase assay using a construct harboring the 3'-UTR of BCL2 (data not shown). Furthermore, no putative binding site for miR-31 is predicted within the 3'-UTR of BCL2 by TargetScan, PicTar, or Miranda target prediction algorithms (27, 29, 30).

Next, we analyzed whether direct down-regulation of BCL2 by RNAi has the potential to induce the phenotypes observed upon overexpression of miR-31 or knockdown of PRKCE. Knockdown of BCL2 significantly increased the level of basal, non-induced apoptosis in MCF10A and in MDA-MB-231 cells (Fig. 5b). In contrast, miR-31 overexpression did not alter sensitivity to chemotherapy (Fig. 5c) or basal apoptosis (Fig. 5d, protein expression as determined by Western blot) of MCF10A cells stably overexpressing the open reading frames encoding PRKCE or BCL2 indicating the importance of 3'-UTR-mediated regulation of PRKCE and consequently BCL2 by miR-31. Furthermore, the effects of miR-31 and siPRKCE on chemoresistance were reproduced by knockdown of BCL2, indicating that indeed down-regulation of BCL2 partially accounts for the sensitization to ionizing radiation (Fig. 5e) or treatment with staurosporine (Fig. 5f) induced by miR-31 over-expression. Taken together, the data suggests that direct targeting of PRKCE and indirect down-regulation of BCL2 at least partially mediate the tumor-suppressive phenotype of miR-31 by sensitizing cells to apoptosis as well as to anti-cancer therapy.

Expression of BCL2 Is Inversely Correlated with miR-31 Expression in Breast Cancer Patients—To address the question whether targeting of PRKCE and the associated down-regulation of BCL2 have functional consequences in breast cancer patients, we analyzed a previously published data set (gene expression omnibus accession no. GSE19783, (31)) comprising matched mRNA and miRNA expression profiles along with clinical information of 99 breast cancer patients for inverse correlations of the expression of miR-31 with the two down-regulated genes PRKCE and BCL2. Our results had indicated that the effect of miR-31 on PRKCE expression is mostly post-transcriptional as suggested by Fig. 2b. Accordingly, we did not observe any correlation between miR-31 and its direct target PRKCE (data not shown). However, expression of the indirectly down-regulated BCL2 gene was significantly and inversely correlated with expression of miR-31, and high expression of BCL2 was associated with poor prognosis (Fig. 5g, Pearson correlation coefficient, r = −0.22, p = 0.03, and Fig. 5h, p = 0.1). This indicates, that expression of miR-31 in breast cancer patients indeed is functionally related to BCL2 expression, likely via direct down-regulation of PRKCE. Thus, this data supports the notion that down-regulation of BCL2 as a result of direct targeting of PRKCE by miR-31 contributes to the complex network regulating breast cancer progression and survival.

DISCUSSION

Many studies have aimed to characterize miRNAs in different cancer entities. Although high expression of miR-31 has previously been linked to poor prognosis in colon, head and neck, and lung cancer, high expression of this miRNA is beneficial in other cancer types including for instance breast cancer, leukemia, and ovarian cancer (18, 19, 32–35). These controversial observations have been linked to the fact that miR-31 targets on the one hand tumor suppressor genes such as the large tumor suppressor, homolog 2 (LATS2) or the factor-inhibiting hypoxia-inducible factor (FIH) (32, 35). Furthermore, suppression of miR-31 in colon cancer cells has been linked to increased sensitivity to the chemotherapeutic drug 5-fluorouracil and impaired cell migration and invasion (34). On the other hand, miR-31 has been shown to directly downregulate expression of various oncoproteins, including the metastasis-promoting genes Ras homolog gene family, member A (RhoA), the Wnt-receptor frizzled 3, radixin, as well as several integrins (18, 36). Additionally, miR-31 expression has been described to be reduced in an
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FIGURE 5. Indirect down-regulation of BCL2 via direct down-regulation of PRKCE accounts for enhanced sensitivity upon overexpression of miR-31. a, mRNA levels of BCL2 upon overexpression of miR-31 or knockdown or PRKCE in MCF10A and MDA-MB-231 cells was quantified by qRT-PCR. Cells were transfected with the indicated miRNA mimics or siRNAs. 48 h after transfection, RNA was extracted, and BCL2 expression was quantified by qRT-PCR. Expression was normalized to the expression of HPRT1 and GAPDH housekeeping genes. A Western blot showing BCL2 protein expression in MCF10A cells upon the indicated transfections is depicted below. b, apoptosis in MCF10A and MDA-MB-231 upon knockdown of BCL2 was assessed by the NucView-488 assay. Cells were transfected with the indicated miRNA mimics or siRNAs. 48 h after transfection, apoptotic cells were stained with NucView-488, and nuclei of all cells were stained using DAPI. Plates were acquired using an Olympus ScanR high-content screening microscope and nuclei as well as cells positive for NucView-488 staining were automatically counted using ScanR analysis software. Similarly, sensitivity to staurosporine and doxorubicin (c) and basal apoptosis (d) were determined using MCF10A cells stably overexpressing BCL2 or PRKCE as indicated. Protein overexpression is depicted next to d, e, radiosensitivity of MCF10A and MDA-MB-231 cells transfected with miRNA mimics and siRNAs was determined by Celltiter Glo cell viability assay 72 h after irradiation. The ratio of the viability of cells irradiated with 5 Gray over the viability of non-irradiated cells is plotted. f, sensitivity of MCF10A cells transfected with the indicated siRNAs to staurosporine was determined by Celltiter Glo cell viability assay 72 h after treatment. Concentrations are plotted on log-10 scale. g, correlation analysis of miR-31 and BCL2 expression in breast cancer patient samples (GSE19783), including clinical data were performed using Pearson correlation ($r = -0.22, p = 0.03, n = 99$). h, Kaplan-Meier plot separating patients (GSE19783) with high BCL2 expression from patients with low BCL2 expression ($n = 99, p = 0.1$). Ctrl, control.
esophageal cancer cell line model for acquired radiosensitivity and re-expression of miR-31 resulted in deregulation of DNA repair genes and resensitization to ionizing radiation of these cells (33). However, the molecular mechanism behind both deregulation of miR-31 expression and sensitization to ionizing radiation remains to be elucidated.

In this study, we characterized miR-31 as a negative regulator of TNF-α-induced NF-κB signaling in both non-tumorigenic breast epithelial MCF10A cells and triple negative MDA-MB-231 breast cancer cells. This regulation was shown to be independent from the previously identified direct targets of miR-31 activating NF-κB activity, RhoA, and NF-κB-inducing kinase.

Down-regulation of NF-κB-inducing kinase by miR-31 had been demonstrated to inhibit NF-κB activity in adult T-cell leukemia (19). NF-κB-inducing kinase is a kinase participating in activation of the non-canonical NF-κB pathway (37). In the same study, increased expression of miR-31 was also linked to impaired NF-κB signaling in breast cancer cells; however, the authors did not elaborate on the causative direct target of miR-31 responsible for reduced NF-κB activity in this cell system (19). In our study, we focused on the canonical, TNF-α-triggered NF-κB pathway. Hence, our observation that down-regulation of NF-κB-inducing kinase does not account for changes in NF-κB activity in our system is in line with the well accepted role of this kinase within the non-canonical, rather than the canonical NF-κB pathway.

In contrast, overexpression of RhoA had been linked to increased activity of the canonical NF-κB pathway, which has been shown to be inhibited by overexpression of dominant-negative mutants of RhoA (38). In our study, siRNA mediated knockdown of RhoA only induced a weak inhibitory effect on TNF-α induced NF-κB activity in HEK293FT, MCF10A, or MDA-MB-231 cells. This indicates that, given the more efficient knockdown of RhoA by siRNA compared with miR-31 overexpression, the inhibitory effect of miR-31 on NF-κB activity must be mediated by an additional direct target exhibiting a stronger phenotype upon knockdown. Therefore, we aimed to identify the direct miR-31 target responsible for the strong inhibition of NF-κB activity.

Using bioinformatic target prediction algorithms, we identified PRKCE as a putative direct target of miR-31 accounting for the observed down-regulation of NF-κB signaling. We validated direct binding of miR-31 to three predicted binding sites within the 3′-UTR of PRKCE resulting in reduced protein expression of PKCe without altering mRNA abundance, indicative of translational repression rather than mRNA degradation as the predominant mechanism of PRKCE suppression by miR-31. Furthermore, we demonstrated that siRNA-mediated knockdown of PRKCE phenocopied the effect of miR-31 overexpression on NF-κB activity, suggesting that direct down-regulation of this target gene at least partly accounts for inhibition of TNF-α-induced NF-κB activity by miR-31. A recent study has investigated the mechanism how PKCe exerts its function of NF-κB activity and shown that it activates the IKKβ kinase, an upstream activator of NF-κB (39).

Because NF-κB-mediated gene expression is a crucial factor influencing cancer cell survival and response to anti-cancer treatment, we then hypothesized that miR-31 overexpression and the resulting down-regulation of PRKCE protein levels might alter these phenotypes. Indeed, both knockdown of PRKCE and overexpression of miR-31 induced apoptosis and increased the sensitivity of MCF10A and MDA-MB-231 cells to both ionizing radiation and treatment with cytostatic compounds. Indeed, we could link these phenotypes to the reduced activity of NF-κB and, importantly, also to impaired transcription of BCL2 in cells with reduced levels of PRKCE. The latter association has been described by Shankar and colleagues (22), showing that PKCe regulates transcriptional activity of the transcription factor cAMP response element-binding protein (CREB), which in turn promotes expression of BCL2. Thus, inactivation of PKCe results in decreased activity of CREB leading to impaired expression of the anti-apoptotic protein BCL2. Thereby, miR-31 facilitates the induction of apoptosis by indirect down-regulation of BCL2. This notion is supported by the observation of an inverse correlation of miR-31 and BCL2 expression in breast cancer patient samples. We found a Pearson correlation of $r = -0.22$ between miR-31 and BCL2. Although a correlation test yields a significant result ($p = 0.03$) on a 5% significance level, in conjunction with our molecular data, we see this rather small correlation value as a trend toward a relationship between miR-31 and BCL2. Importantly, high expression of BCL2 was associated with poor prognosis in these patients, further highlighting the clinical relevance of this indirect down-regulation.

Lynam-Lennon et al. (33) reported that miR-31 expression is reduced in radioresistant esophageal cancer cells and re-expression of miR-31 sensitized these cells to ionizing radiation. The miR-31-induced down-regulation of a number of genes involved in DNA damage response led them to propose this as the mechanism mediating the miR-31-associated sensitization to ionizing radiation. However, our study suggests that direct down-regulation of PRKCE and the resulting reduction in NF-κB activity as well as the indirect down-regulation of BCL2 expression may contribute to radiosensitivity. However, further experimental validation is necessary to prove this hypothesis.

In summary, we have identified PRKCE as a novel direct target of miR-31. Functionally, we linked this targeting to reduced NF-κB activity and enhanced susceptibility of breast cell lines to apoptosis as well as sensitization to ionizing radiation and cytotoxic drugs. As a molecular mechanism behind the described sensitization, we propose the indirect down-regulation of BCL2 by suppression of PKCe expression by miR-31. In clinical samples from breast cancer patients, we observed an inverse correlation between the expression of miR-31 and its indirect target BCL2 as well as an association of high BCL2 expression with poor prognosis. Taken together, these data suggest that miR-31 does not only inhibit breast cancer metastasis as previously reported but also proliferation.

Furthermore, triple negative breast cancer lacks molecular targets is exceptionally challenging with respect to therapeutic options. Hence, a combined therapeutic approach involving classical chemotherapy along with a pleiotropically acting
miRNA such as miR-31 targeting cancer cell proliferation, metastasis, and resistance collectively might be superior to current anti-cancer treatments.

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