miR-106b~25 cluster regulates multidrug resistance in an ABC transporter-independent manner via downregulation of EP300

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Abstract. MicroRNA (miR)-106b~25 cluster regulates bypass of doxorubicin and γ-radiation induced senescence by downregulation of the E-cadherin transcriptional activator EP300. We asked whether upregulation of miR-106~25 cluster generates cells with a truly multidrug resistant (MDR) phenotype and whether this is due to upregulation of the ATP-binding cassette (ABC) transporter P-glycoprotein. We used minimally transformed mammary epithelial breast cancer cells (MTMECs) in which the miR-106b~25 cluster was experimentally upregulated by lentiviral transfection or in which hairpins targeting either EP300 or E-cadherin mRNAs have been expressed with lentiviruses. We find that overexpression of miR-106b~25 cluster led to the generation of MDR MTMECs (resistant to etoposide, colchicine and paclitaxel). Paclitaxel resistance was also studied after experimental downregulation of EP300 or E-cadherin. However none of these cells overexpressed P-glycoprotein or were able to efflux a fluorescent derivative of paclitaxel, making this phenotype drug-transporter independent. Paclitaxel treatment in MTMECs led to an increase in early apoptotic cells (Annexin V-positive), activation of caspase-9 and increase in the proportion of cells at the G2/M phase of the cell cycle. However, MTMEC overexpressing miR-106b~25 cluster, or with EP300 or E-cadherin downregulated, showed less activation of apoptosis, caspase-9 and caspase-3/-7 activities. Thus, miR-106b~25 cluster controls transporter-independent MDR by apoptosis evasion via downregulation of EP300.

Introduction

Chemotherapy is widely used for cancer treatment. Unfortunately, in a high proportion of cases, cancer cells become insensitive to the cytotoxic drugs and are able to proliferate and metastasize, normally with fatal consequences. How cancer cells acquire drug resistance remains to be fully understood, although the effector molecules and pathways involved in the process are relatively well characterized. Cancer cells can increase the activity of DNA repair enzymes, metabolize drugs or activate anti-apoptotic mechanisms (1), although the most common and best studied mechanism of drug resistance is the upregulation of membrane transporters (2). ABC transporters are membrane-anchored proteins that use the energy from ATP hydrolysis to mediate the export of cytoplasmic or membrane solutes outside of the cell (3) and have a broad substrate specificity. This leads to MDR, by which cells become insensitive to structurally and mechanistically unrelated cytotoxic drugs (4). In the clinic, resistance due to P-glycoprotein (ABCB1 or MDR1) has been demonstrated in leukemias (5) and breast cancer (6,7).

MicroRNAs (miRs) are a class of 18- to 24-nucleotide single-stranded non-coding RNAs as negative regulators of
gene expression by triggering translation repression through partial complementation to 3'-untranslated region (UTR) of target mRNAs (8). miRs play crucial roles in multiple biological processes, including cancer (9,10) and drug resistance. miR-214 confers cell survival and cisplatin resistance in ovarian cancer cells (11); miR-125b confers the resistance of breast cancer cells to paclitaxel (12) and miR-221/222 confers tamoxifen resistance in breast cancer (13).

The miR-106b~25 cluster, consisting of miR-106b, miR-93 and miR-25, is highly conserved in vertebrates, and is located in intron 13 of the minichromosome maintenance complex component 7 (MCM7) oncogene. It may play an important proto-oncogenic role in cellular transformation and tumorigenesis by downregulation of several tumor suppressors such as p21, E2F1, Bim and PTEN (14-16). We have recently reported that the miR-106b~25 cluster negatively regulates the histone acetyltransferase EP300, a transcriptional activator of E-cadherin. This leads to activation of an epithelial-to-mesenchymal transition (EMT), increase in the ability of cell migration and invasion, and resistance to doxorubicin and γ-radiation (17).

Here we report that breast cancer cells overexpressing miR-106b~25 cluster, or in which EP300 or E-cadherin have been downregulated by RNA interference, have a P-glycoprotein-independent (transporter independent) MDR phenotype that involves apoptosis evasion.

Materials and methods

Cells. Minimally transformed mammary epithelial cells (MTMECs) overexpressing the miR-106b~25 cluster by lentiviral transfection (MTMEC-miR-106b~25) or expressing a short hairpin targeting EP300 or E-cadherin (MTMEC-shEP300 and MTMEC-shCDH1, respectively) mRNAs have been described (17). MTMECs, that are human mammary epithelial primary cells that have been transformed experimentally and express TERT, SV40 large T antigen, a constitutively active form of PI3K, p110α, and oncogenic ras (18), were routinely cultivated on serum-free HuMEC medium (Life Technologies). The multidrug resistant cell line NCI/ADR-Res (19) and P-glycoprotein-negative CAL51 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/l glucose, 10% foetal calf serum and 4 mM L-glutamine (Invitrogen) in the presence or absence of 1 μM doxorubicin, respectively.

Drug resistance clonogenic assay. MTMEC-derived cells were seeded, at least in duplicate, at a density of 3x10^5 cells in 25-cm² culture flasks and exposed to a single dose of drug for 3 days. Cells were kept in culture for 21 days with drug-free medium changes every three days. Drug resistant clones were fixed with 4% paraformaldehyde and stained with 0.2% crystal violet and counted.

Drug efflux assay. Functional drug efflux assays were performed using 0.1 μM BODIPY-paclitaxel (Invitrogen) in the presence or absence of 1 μM cyclosporin A (Sigma-Aldrich) essentially as described (20,21) by flow cytometry in a Becton Dickinson FACSDiva. Briefly, cells were detached from the culture dishes with 10 mM EDTA and washed with phenol red-free DMEM containing 0.1% bovine serum albumin (DMEM-BSA) and 2x10⁶ cells were stained with either 0.1 μM BODIPY-paclitaxel, 0.1 μM BODIPY-paclitaxel containing 1 μM cyclosporin A to inhibit ABC transporter efflux, or left untreated to determine auto-fluorescence. After 30 min at 37°C, cells were washed with DMEM-BSA and dead cells were stained with TOTO3-iodide (Life Technologies) and gated out.

P-glycoprotein expression assay. Cell surface P-glycoprotein (ABCB1) was determined by flow cytometry in a Becton Dickinson FACSDiva using the phycoerythrin-conjugated UIC2 antibody (Immunotech, Marseille, France) or the corresponding isotype control (Sigma-Aldrich) essentially as described (22). Briefly, cells were detached from the culture dishes with 10 mM EDTA and washed with DMEM-BSA. Antibodies (250 ng) and cells (5-10⁵) were incubated for 30 min at 37°C in the presence of 1 μM cyclosporin. Cells were then washed with DMEM-BSA and dead cells were stained with TOTO3-iodide and gated out.

Gene expression analysis. For mRNA detection, total RNA (isolated using a miRCURY RNA isolation kit; Exiqon) was reverse transcribed with RNase H+ MMLV reverse transcriptase (iScript cDNA Synthesis kit) and real-time quantitative PCR was performed using SYBR-Green (Bioline) and ABCB1 specific primers (19) on an ABI Prism 7700 detection system (PerkinElmer Life Sciences). A comparative threshold cycle was used to determine the relative gene expression using two normalizers, RPS6 and RPS4 as previously described (22,23).

Apoptosis. Apoptotic assessment was by detection of active caspase-9 and -3/-7 using Caspase-Glo assays (Promega) following the manufacturer's protocol. Caspase activity was normalized to cell density determined by sulphorhodamine B (Sigma-Aldrich) staining (24). For cell cycle analysis cells were stained with propidium iodide after fixation with ice-cold 70% methanol and the DNA content estimated determined by flow cytometry essentially as described (23). Annexin V staining was determined by flow cytometry using an Annexin V-FITC apoptosis detection kit (BioVision) as described (25).

IC₅₀. The drug concentration necessary to kill 50% of cells (IC₅₀) was obtained after sulphorhodamine B (Sigma-Aldrich) staining (24) as previously described (26).

Statistical analysis. Statistical evaluations were performed by Student's t-test for paired data, and data were considered significant at a p-value <0.05.

Results

Overexpression of the miR-106b~25 cluster leads to a MDR phenotype. We have recently reported that experimental upregulation of the miR-106b~25 cluster in MTMEC cells leads to the acquisition of doxorubicin and γ-radiation resistance (17). We asked whether upregulation of this miR cluster would lead to resistance to other structurally unrelated drugs. First, we tested etoposide, that, as doxorubicin, is a topoisomerase II inhibitor (27). Indeed, MTMEC cells

HU et al: TRANSPORTER-INDEPENDENT MULTIDRUG RESISTANCE 1171
overexpressing the miR-106b~25 cluster generated a higher number of etoposide-resistant clones than control cells transfected only with the empty vector (Fig. 1). Next we asked whether overexpression of the miR-106b~25 cluster would also influence the generation of resistance to other drugs with different modes of action. For this we selected two microtubule interfering agents, colchicine and paclitaxel. Long-term clonogenic assays indicated that indeed MTMEC cells overexpressing miR-106b~25 cluster generated more colchicine- and paclitaxel-resistant clones than control cells transfected only with empty vector (Fig. 1).

Doxorubicin, at low to moderate concentrations, and \( \gamma \)-irradiation trigger a senescent phenotype, very similar to the well-characterized replicative senescence, often termed drug (or therapy)-induced senescence (28). Experimental upregulation of miR-106b~25 cluster in MTMEC cells allows cells to bypass senescence and to proliferate after doxorubicin or \( \gamma \)-irradiation treatment, becoming resistant (17). However, other drugs, such as the taxanes paclitaxel and docetaxel, exert their cytotoxic effect by kinetic suppression of microtubules that block cells in the G2/M phase of the cell cycle and trigger apoptosis (29). When MTMEC cells were treated with increasing concentrations of paclitaxel there was a dose-dependent increase in Annexin V staining and activation of caspase-9 (Fig. 2). This confirms that paclitaxel, as expected, acts on breast cancer cells triggering an apoptotic programme.

Thus, upregulation of miR-106b~25 cluster confers cells the ability to generate resistance to a variety of structurally and mechanistically different drugs, a hallmark of MDR (4).

Downregulation of EP300 leads to the MDR phenotype. The three miRs in the miR-106b~25 cluster bind EP300 3'-UTR mRNA leading to its downregulation, decreasing E-cadherin levels and activating an EMT accompanied by resistance to
doxorubicin and γ-irradiation in MTMECs (17). As resistance to doxorubicin and γ-irradiation can be mimicked by experimental downregulation of EP300, we asked whether the same would apply to paclitaxel. Short-term drug sensitivity assays indicated that MTMECs overexpressing miR-106b~25 were slightly more resistant to paclitaxel than control cells (IC50 values of 30 and 22 nM, respectively). In MTMECs with EP300 downregulated by RNA interference the paclitaxel IC50 had increased to 60 nM (~3-fold; Fig. 3A). Long-term generation of paclitaxel resistance was also affected. When MTMEC-shEP300 cells were treated with paclitaxel, a large number of drug-resistant proliferating clones were generated whereas this was not the case with control cells (Fig. 3B and C). Thus, downregulation of EP300 leads to a decrease in paclitaxel sensitivity and generation of paclitaxel-resistant cells.

The above, and our previously published data, indicate that downregulation of EP300 leads to the MDR phenotype. ABC transporters are not responsible for the MDR phenotype of cells either overexpressing the miR-106b~25 cluster or with EP300 downregulated. Upregulation of ABCB1 (P-glycoprotein) is the main mechanism by which cells become multidrug resistant. To test whether the multidrug resistant phenotype of MTMEC-miR-106b~25 and MTMEC-shEP300 cells was due to ABCB1, we tested first ABCB1 mRNA levels by RT-QPCR including NCI-ADR/RES and CAL51 as positive and negative controls, respectively. ABCB1 mRNA levels in CAL51 were between 1,000 and 10,000 lower than those found in ABCB1-positive NCI-ADR/RES cells (Fig. 4A). Importantly, ABCB1 mRNA expression in all MTMECs was one order of magnitude lower than in CAL51 cells (Fig. 4A).

As we have previously demonstrated that ABCB1 mRNA and protein levels do not correlate (22), we determined functional ABCB1 by flow cytometry using UIC2 antibody (23) and NCI-ADR/RES and CAL51 cell lines as positive and negative controls, respectively. None of the MTMECs showed an increase in fluorescence after ABCB1-specific antibody binding with respect to the isotype IgG control (Fig. 4B). These results indicate that MDR in MTMECs is not due to upregulation of ABCB1.

As several other transporters can be responsible for multidrug resistance (30), we asked whether MTMECs were able to efflux paclitaxel out of the cell. For this, we took advantage of BODIPY-paclitaxel, a drug-derivative with a fluorescent dye used to determine efflux pump activity (23). Importantly, all MTMECs, as well as the negative cell line CAL51, were unable to efflux BODIPY-paclitaxel (Fig. 4C). In contrast, NCI-ADR/RES cells showed, as expected, efflux activity that was inhibited by cyclosporin A (Fig. 4C).

In summary, the multidrug resistance phenotype of MTMECs, either overexpressing miR-106b~25 or with EP300 downregulated, is not due to transporter activity.
asked whether MDR MTMECs have altered cell cycle profiles. After treating cells with 20 nM paclitaxel for 48 h MTMEC-ev control cells showed, as expected, a decrease in the percentage of cells at the G1 phase of the cell cycle and an increase in G2/M (from 67 to 52% in G1 and from 13 to 29% in G2/M). The percentage of cells in S was practically the same (Fig. 5). However, the percentage of cells in G2/M after paclitaxel treatment increased only by 5% in MTMEC-miR-106b−25 cells (from 10 to 15%) and was practically the same in MTMEC-shEP300 cells (from 14 to 15%). Thus, the cell cycle effects of paclitaxel are abolished in MDR MTMECs.

Figure 3. Downregulation of EP300 leads to paclitaxel resistance. (A) Drug sensitivity curves of MTMECs treated with paclitaxel for 72 h determined by sulphorhodamine B staining. The IC50 of control cells (MTMEC-ev) and those overexpressing miR-106b−25 cluster (MTMEC-miR-106b−25) or with EP300 downregulated (MTMEC-shEP300) is indicated in the abscissa. (B) Cells were treated for 3 days with paclitaxel and drug-resistant clones were stained with crystal violet after 3 weeks (upper panel) and number of clones counted (lower panel). Numerical data represent the average ± SD of at least three different experiments (*P<0.05). A representative picture of paclitaxel-resistant clones is shown.

Figure 4. ABC transporters are not responsible for the MDR phenotype of cells either overexpressing miR-106b−25 cluster or with EP300 downregulated. (A) ABCB1 mRNA expression was determined by RT-QPCR and was normalized to RPS6 and RPS9 mRNA expression. Relative ABCB1 expression in the ABCB1-positive cell line NCI-ADR/RES was set up at 100. CAL51, an ABCB1-negative cell line, was used as a negative control. Data represent the average ± SD of at least three different experiments (*P<0.05). (B) Flow cytometric analysis of ABCB1 expression using the phycoerythrin-conjugated UIC2 antibody (black peaks) and the corresponding IgG isotype control (grey peaks). NCI-ADR/RES cells were used as positive and CAL51 as negative controls. (C) Flow cytometric analysis of BODIPY-paclitaxel efflux. The incubation of cells in the presence of a membrane pump inhibitor (cyclosporin A, white peaks) produces an increase in the fluorescence of the cells since they cannot extrude the fluorescent dye out of the cells. AF, autofluorescence. For CAL51, negative control, and MTMECs the fluorescence peaks in the presence and absence of cyclosporin (grey peaks) do overlap, indicating absence of cyclosporin-inhibitable membrane pumps. Flow cytometry was performed in triplicate and one representative plot is shown.
Thus, overexpression of miR-106b–25, or downregulation of EP300 (a direct target of the three miRs in the cluster), leads to a transporter-independent MDR phenotype involving apoptosis evasion.

**Downregulation of E-cadherin leads to the MDR phenotype which is transporter-independent.** We have previously determined that doxorubicin resistance in MTMECs due to overexpression of miR-106b–25 cluster, or to downregulation of EP300, can be mimicked by experimentally downregulating E-cadherin expression (17). As the three miRs in the miR-106b–25 cluster target EP300 downregulating its expression and EP300 is a transcriptional activator of E-cadherin, we asked whether the experimental downregulation of E-cadherin would also lead to paclitaxel resistance. Indeed, MTMEC-shCDH1 cells led to a slightly higher number of paclitaxel resistant clones (Fig. 7A) than MTMEC-miR-106b–25 (Fig. 1) and MTMEC-shEP300 cells (Fig. 3B). Short-term paclitaxel sensitivity was also lower in E-cadherin knockdown cells (IC$_{50}$ ~100 nM; Fig. 7B) than in those with the miR-106b–25 upregulated (IC$_{50}$ ~30 nM) or EP300 downregulated (IC$_{50}$ ~60 nM; Fig. 3A). This indicates that MTMEC-shCDH1 cells have an MDR phenotype despite not having overexpression of ABCB1 and not transporting paclitaxel out of the cells (Fig. 7C). However, activation of caspase-9 and caspase-3/-7 in response to paclitaxel was lower in cells with E-cadherin knocked down (Fig. 7D). Thus, transporter-independent MDR can be acquired by downregulation of E-cadherin via apoptosis evasion.

**Discussion**

Here we report that the axis EP300–E-cadherin, which is controlled by the miR-106b–25 cluster, regulates paclitaxel resistance in breast cancer cells by apoptosis evasion. This pathway also determines the resistance to DNA damaging agents, such as doxorubicin or $\gamma$-radiation, bypassing therapy-induced senescence, hallmarks of MDR. This phenotype was independent of membrane pumps, but involved apoptosis evasion. Thus, transporter-independent MDR can be generated by modulation of the miR-106b–25 cluster–EP300–E-cadherin pathway.

Resistance to chemotherapeutics used in cancer therapy remains one of the main hurdles to overcome for the successful treatment of this disease. Although in many cases the initial response to chemotherapy is positive, in a high proportion of cases, and after a disease-free period, resistant cells give rise to secondary tumors normally at distant sites following metastasis, normally with fatal consequences (32). The use of combination therapies aims to overcome resistance to single agents. However, drug resistant cancer cells develop many times acquiring decreased sensitivity to a broad spectrum of structurally and functionally different drugs, a phenomenon early recognized and termed MDR (33). During tumor progression, epithelial cells lose polarity and acquire characteristics of mesenchymal cells including the capacity to invade surrounding tissues. This EMT is normally accompanied by an increase in the stem cell population, the so-termed cancer stem cells, and acquisition of drug resistance (34). We have recently demonstrated that a cluster of three miRs (miR-
Figure 6. Paclitaxel resistance in cells overexpressing miR-106b~25 cluster, or with downregulation of EP300, is due to apoptosis evasion. (A) Detection of apoptotic cells by Annexin V staining. MTMECs were treated with paclitaxel for 48 h and stained with propidium iodide and Annexin V-FITC prior to flow cytometry. Number of apoptotic cells (Annexin V-positive cells, both in early apoptosis, lower right quadrant, and late apoptosis, upper right quadrant) decreased in both MTMEC-miR-106b~25 and MTMEC-shEP300 cells. A representative dot plot is shown on the left. (B) Activation of both caspase-3/-7 and -9 was determined after paclitaxel treatment for 48 h. Data are normalized to cell density determined by sulphorhodamine B staining. Numerical data represent the average ± SD of at least three different experiments (*P<0.05).

Figure 7. Downregulation of E-cadherin leads to a MDR phenotype which is transporter-independent. (A) MTMEC-shCDH1 are able to generate paclitaxel-resistant clones. Cells were treated for 3 days with paclitaxel and drug-resistant clones were stained with crystal violet after 3 weeks (left panels) and number of clones counted (right panels). MTMECs transfected with empty vector (MTMEC-ev) were used as controls. (B) Paclitaxel IC50 of MTMECs treated with paclitaxel for 72 h was determined by sulphorhodamine B staining. (C) Flow cytometric analysis of ABCB1 expression using the phycoerythrin-conjugated UIC2 antibody (upper panel) and BODIPY-paclitaxel efflux (lower panel) in MTMEC-shCDH1 and control MTMEC-ev cells. (D) Activation of both caspase-3/-7 and -9 was determined after paclitaxel treatment for 48 h. Data are normalized to cell density determined by sulphorhodamine B staining. Numerical data represent the average ± SD of at least three different experiments (*P<0.05). Pictorial data show representative paclitaxel resistant clones and flow cytometry histograms.
106b, miR-93 and miR-25) negatively regulates the expression of EP300, a histone acetyltransferase that transcriptionally activates E-cadherin, leading to an increase in motility and invasion and doxorubicin and γ-irradiation resistance in breast cancer cells (17). Loss of E-cadherin constitutes one of the hallmarks of the EMT process and negative regulators of E-cadherin are well studied (35,36). Loss of functional E-cadherin renders cells more resistant to paclitaxel (37) and downregulation of EP300 (both experimental and in drug resistant lines) is associated with doxorubicin and cisplatin resistance in bladder cancer cells (38,39) and metastatic properties in pancreatic cancer (40). Here we demonstrate that minimally transformed mammary epithelial cells in which the miR-106b-25 cluster is upregulated, or in which either EP300 or E-cadherin have been downregulated by RNA interference (17) are able to generate paclitaxel resistance and are thus MDR.

There are several effectors of MDR, although the most common is upregulation of drug transporters such as ABCB1 (41). MDR has also been associated with E-cadherin loss (42,43). However, none of the MDR cells in this study upregulate ABCB1 mRNA or functional ABCB1 at the cell surface. Other ABC transporters, such as ABCG2, which is frequently found upregulated in cancer stem cells (44), are not responsible for the MDR phenotype as are not able to efflux a fluorescent derivative of paclitaxel. Thus, MDR controlled by the miR-106b-25 cluster via downregulation of EP300 and E-cadherin is transporter-independent.

Paclitaxel, as well as docetaxel, the other taxane currently used in the clinic, is a microtubule-stabilizing agent that interferes with spindle microtubule dynamics causing cell cycle arrest and apoptosis (29). Although some studies indicate a mode of action via the extrinsic apoptotic pathway (45,46), the mitochondrial pathway is clearly involved as caspase-9 activation has been unequivocally demonstrated (47). In addition, paclitaxel has also been shown to activate caspase-2 (48). MTMECs have p53 inactivated due to expression of SV40 large T (18). Thus, the caspase activation observed following paclitaxel treatment must be p53-independent. Although p53 is normally associated with the cytochrome c release from the mitochondria and activation of caspase-9, this can also occur in a p53-independent manner (49). Our data also indicates activation of caspase-9, although we cannot rule out the involvement of a non-mitochondrial pathway. However, although the initiator signals triggering apoptosis may not have been fully elucidated yet, these ultimately converge into executioner caspases, such as caspase-3/-7. Indeed, overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cells with acquired resistance to epirubicin, etoposide and paclitaxel (31). Importantly, we show here that upregulation of miR-106b-25 cluster, or downregulation of either EP300 or E-cadherin, leads to a lower activation of caspase-3/-7 than in control cells, upon paclitaxel treatment.

The MDR phenotype can be fully mimicked by experimental downregulation of E-cadherin in MTMECs. However, we cannot rule out the possibility that either the miRs in the miR-106b-25 cluster, or EP300, or both, act on downstream molecules that regulate themselves drug resistance. miRs act repressing the expression of hundred of targets and EP300, which as a transcriptional co-activator, can also affect expression of many genes. This offer the prospect of finding novel molecules and regulatory pathways controlling transporter-independent MDR.

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