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Regulation of MDR1 gene expression in multidrug-resistant cancer cells is independent from YB-1

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Abstract

The MDR1 gene encoded transmembrane ABC-transporter MDR1/P-glycoprotein can mediate the phenotype of multidrug resistance (MDR), a major obstacle in the clinical management of cancer patients. It was hypothesized that YB-1 is a fundamental regulatory factor of the MDR1 gene in tumor cells and can therewith enhance drug resistance. To analyze the potential impact of YB-1 in MDR cancer cells, two specific anti-YB-1 small interfering RNAs (siRNAs) were designed for transient triggering the gene-silencing RNA interference (RNAi) pathway in the MDR cell lines EPG85-257RDB and EPP85-181RDB as well as in their drug-sensitive counterparts EPG85-257P and EPP85-181P. Since both siRNAs showed biological activity, for stable inhibition of YB-1 corresponding tetracycline-inducible short hairpin RNA (shRNA)-encoding expression vectors were designed. By treatment of the cancer cells with these constructs, the expression of the targeted YB-1 encoding mRNA and protein was completely inhibited following tetracycline exposure. These gene-silencing effects were not accompanied by modulation of the MDR1 expression or by reversal of the drug-resistant phenotype. In conclusion, the data demonstrate the utility of the analyzed RNAs as powerful laboratory tools and indicate that YB-1 is not involved in the regulation of the MDR1 gene or the development of the drug-resistant phenotype in MDR cancer cells.

Keywords: Multidrug resistance; YB-1; MDR1; RNAi; Gene regulation

The ABC-transporter MDR1/P-glycoprotein (MDR1/P-gp; ABCB1) was the first identified factor that mediates multidrug resistance (MDR) to tumor cells [1]. The MDR phenotype, the simultaneous resistance against various anticancer agents with different structures and modes of action, is a common problem in patients with cancer and a poses major obstacle to effective treatment of disseminated neoplasms. Different regulatory mechanisms controlling the expression of the MDR1/P-gp encoding MDR1 gene have been identified [2].

Like all of the human drug-related ABC-transporter genes, the MDR1 promoter lacks an appropriately positioned TATA box. Instead, the MDR1 promoter includes an inverted CCAAT box [3], that can potentially interact with the regulatory protein YB-1 [4] or the transcription factor NF-Y [5]. Previous studies implicated YB-1 as a fundamental regulatory factor for the MDR1 gene in human cancers [6]. In vitro experiments with MDR1 promoter-depending reporter gene constructs demonstrated that YB-1 could activate the expression of the reporter constructs [4,7]. Furthermore, nuclear YB-1 expression was demonstrated to be associated with enhanced MDR1 gene expression and drug resistance in clinical specimens of cancer patients [6,8–11]. However, a direct experimental proof that YB-1 can activate the transcription of the cellular MDR1 gene and not of artificial MDR1 promoter constructs is missing. Not even by transfection of the YB-1 encoding cDNA, an elevated transcription rate of the endogenous MDR1 gene could be observed [12].

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Thus, in this study a very efficient RNA interference (RNAi)-mediated knock down of YB-1 expression in MDR cells was developed. The experiments were performed transiently by using chemically synthesized anti-YB-1 siRNAs and stably by treatment of cancer cells with anti-YB-1 short hairpin RNA (shRNA)-encoding tetracycline-inducible expression vectors.

Material and methods

Cell lines and cell culture. Establishment and cell culture of the human gastric carcinoma cell line EPG85-257P [13] and the human pancreatic carcinoma cell line EPP85-181P [14] were described in detail previously. The "classical" multidrug-resistant MDR1/P-gp-positive derivatives EPG85-257RDB [15] and EPP85-181RDB [14], were established by in vitro exposure to daunorubicin (Farmitalia Carlo Erba, Freiburg, Germany). The cell lines were grown in modified Leibovitz L15 medium as described previously [13,14].

Design and application of anti-YB-1 siRNAs. Two different YB-1-specific siRNA duplexes were designed to be homologous to the YB-1-encoding mRNA consensus sequence (GenBank Accession No. NM_004559.2). The target sequences of two different siRNA molecules, Y1 (5′-GAAGGUCAUCGCAACGAAG-3′) and Y2 (5′-GGAAGAU GUAUUUGUACAC-3′), homologous to nt 269–287 and nt 356–374 of sequence NM_004559.2 were chosen according to general recommendations [16,17, http://www.ambion.com/techlib/misc/siRNA_finder.html]. As control, a siRNA molecule specific for luciferase (Luc: 5′-CGUACGC GGAAUACUUCGA-3′) was used [18]. The 21-nt siRNAs contained 3′-dTdT extensions and were commercially obtained from Dharmacon (Lafayette, CO, USA). Transfection of human gastrointestinal carcinoma cells with siRNAs was performed using Oligofectamine Reagent (Invitrogen, Carlsbad, CA, USA) as described previously [19–21].

Anti-YB-1 shRNA design and construction of inducible anti-YB-1 shRNA expression vectors. Anti-YB-1 shRNA sequences were chosen to be homologous to the chemically synthesized anti-YB-1 siRNAs, Y1 and Y2, demonstrated for their gene-silencing activity. As control, the sequence of the anti-luciferase siRNA Luc was selected. In each case, homologous single-stranded DNA (ssDNA) molecules were chemically synthesized. Annealing of the ssDNA molecules was performed by incubation of 2.5 μM of each complementary ssDNA oligonucleotide in 0.15 M NaCl in a total volume of 20 μl. The annealing mixture was incubated at 80 °C for 2 min followed by cooling to 35 °C. The annealed dsDNA, consisting of the sense and antisense sequences, spacer sequences [20–22], and a 5T termination signal were site directed cloned into the *Bgl*II and *Hin*dIII double restricted expression vector pTER [23] containing a modified RNA Polymerase III-depending H1-RNA promoter with a tetracycline operator region. In cells engineered to express the bacterial tetracycline repressor, this vector does not express anti-YB-1 shRNAs until a tetracycline is added. The correct insertion of the specific shRNA-encoding DNA molecules was confirmed by sequencing.

Establishment of tetracycline repressor expressing cancer cells. The human gastric carcinoma cell line EPG85-257P and pancreatic carcinoma cell line EPP85-181P as well as their classical MDR variants EPG85-257RDB and EPP85-181RDB were transfected with 2 μg the tetracycline repressor encoding expression vector pcDNA6/TR (Invitrogen) using transfection reagent SuperFect (Qiagen, Hilden, Germany) to generate corresponding tetracycline repressor expressing clones EPG85-257P/tetR, EPP85-181P/tetR, EPG85-257RDB/tetR, and EPP85-181RDB/tetR. The transfected cell clones were selected in blasticidin-containing (10 μg/ml) cell culture medium. The efficacy of the tetracycline-dependent gene expression was measured by luciferase activity using standard procedures [24].

Anti-YB-1 shRNA expression vector transfection. Tetracycline repressor expressing clones EPG85-257P/tetR, EPP85-181P/tetR, EPG85-257RDB/tetR, and EPP85-181RDB/tetR were transfected with 2 μg of shRNA encoding expression vector DNA as described above. In addition to 10 μg/ml blasticidin, 200 μg/ml Zeocin (Invitrogen) was added to select cell clones which had been stably transfected with both vectors. 2 μg/ml tetracycline (doxycycline, Invitrogen) was added to induce anti-YB-1 shRNA expression.

Quantitative RT-PCR. For quantitative mRNA expression analysis, a real-time RT-PCR protocol was applied using a LightCycler instrument and SYBR-Green Fluorescent dye (Roche Diagnostics, Mannheim, Germany) as described previously [25]. For quantification of YB-1 and MDR1 mRNA expression levels, transcripts levels were normalized using 18S rRNA as an internal control. Specific oligodeoxynucleotide primers used for amplification of each target were as follows: 18S rRNA-fw 5′-GAT ATG CTC ATG TGG TGT TG-3′ and 18S rRNA-rev 5′-AAT CTT CTT CAG TCG CTC CA-3′; YB-1-fw 5′-CAA TGT AAG GAA CGG ATA TGG-3′ and YB-1-rev 5′-TTC CCC ACT CTC ACT ATT CTG-3′; MDR1-fw 5′-CAG CTA TTC GAA GAG TGG GC-3′ and MDR1-rev 5′-CCT GAC TCA CCA CAC CAA TG-3′, yielding expected amplification products of 236 bp (18S rRNA), 310 bp (YB-1 mRNA), and 299 bp (MDR1 mRNA). Cycling conditions were as follows: initial enzyme activation at 95 °C for 10 min, followed by 40 cycles of 15 s denaturation at 95 °C, 5 s annealing at 55 °C (YB-1), 58 °C (18S rRNA, MDR1), respectively, 10 s elongation at 72 °C. Specificity of amplification products was confirmed by melting curve analysis (T_m 18S rRNA, 84 °C; T_m YB-1: 84 °C; T_m MDR1, 84 °C). Quantitative measurements of each mRNA expression were repeated three times. Levels of statistical significance were evaluated by calculation of the two-tailed P-value by performing the unpaired *t*-test.

Northern blot analysis for detection of YB-1 and MDR1 mRNA. The amount of the YB-1 or MDR1 encoding mRNAs was determined by Northern blot analysis applying standard procedures as described previously [19,22,25]. Blots were hybridized with 25 ng of a YB-1 or MDR1-encoding cDNA fragment labeled with [^{32}P]dCTP by random primed labeling (Amersham). As control for equal RNA loading the membranes were stripped and rehybridized with a fructose-bisphosphate aldolase specific cDNA probe.

Western blot analysis for detection of YB-1 and MDR1/P-gp protein. Cellular content of YB-1 and MDR1/P-gp, was detected by Western blot as described previously [19,22,25]. Proteins were detected using polyclonal rabbit antibodies against YB-1 [6], kindly provided by Dr. Karsten Jürchott (Charité, Institute of Pathology, Berlin, Germany), and MDR1/P-gp (mAb C219; Alexis, San Diego, CA, USA) at 1:1000 and 1:100, respectively. As a loading control, mouse mAb directed against actin (mAb 1501 R; Chemicon, Temecula, CA) was used at 1:5000. Two hours primary antibody incubation were followed by incubation with peroxidase-conjugated mouse anti-rabbit IgG (1:10,000) (A-1949; Sigma, St. Louis, MO, USA) and the protein–antibody complexes were visualized by chemiluminescence developing reagents (ECL system, Amersham plc, Buckinghamshire, UK). Each Western blot is representative of at least three independent experiments.

Cell proliferation assay. Chemosensitivity against anthracycline treatment was determined using a cell proliferation assay based on sulphorhodamine B (SRB) staining. Transient siRNA transfected cells as well as stable shRNA expressing cell variants were analyzed as described in detail previously [14,19–22,25]. In each case, IC_{50} values were calculated from at least three independent experiments in triplicate.

Results and discussion

Modulation of YB-1 mRNA expression by siRNAs

Two different siRNA constructs, Y1 and Y2, were used to decrease the mRNA expression level of YB-1 in the "classical" multidrug-resistant gastrointestinal cancer cell lines EPG85-257RDB and EPP85-181RDB. Both siRNA sequences were designed according to general rec-

ommendations of siRNA selection. Northern blot experiments were performed to demonstrate the gene-silencing activity of the chosen siRNA molecules (Fig. 1). Application of both siRNAs achieved a considerable decrease of the ABCC2 mRNA expression level. The extent of the gene-silencing activity was comparable to other well characterized siRNAs directed against alternative targets in these cell models [19,21].

Anthracycline sensitivity of anti-YB-1 siRNA treated cancer cells

Anti-YB-1 siRNA treated cancer cells were analyzed concerning potential alterations in the sensitivity against the anthracycline daunorubicin, a classical MDR1/P-gp substrate. In three independent experiments neither parental, drug-sensitive cells, nor multidrug-resistant MDR1/P-gp-positive cancer cells, showed an altered drug sensitivity (Table 1) when YB-1 expression was silenced. These data indicate that YB-1 has no impact on the drug-resistant phenotype or on MDR1/P-gp expression.

Some investigators have interpreted an influence of YB-1 on the expression of alternative anthracycline-transporting ABC-transporters like MRP1 or BCRP [7,12]. Thus, the down regulation of YB-1 could also affect these drug extrusion pumps. However, previous gene expression analyzes showed that the used gastrointestinal cancer cell models do not over express these anthracycline pumps [14,15]. Therewith, in the investigated cell models a regulatory control of YB-1 on these transmembrane transporter proteins can be excluded.

On the other hand, a potential YB-1-depending effect on ABC-transporter expression or drug resistance could be a time-consuming effect, e.g. due to long biological half-live times of the involved proteins. Thus, a stable anti-YB-1 shRNA expression system was established. Data are available that enhanced YB-1 expression could show anti-apoptotic activity [26]. Furthermore, antisense oligonucleotide-mediated down regulation of YB-1 in cancer cells could induce p53 activity and, therewith, trigger programmed cell death [27]. In the case of that event it would not be possible to select anti-YB-1 shRNA expressing cell clones; such a clone would die by apoptosis. Thus, a tetracycline-dependent inducible anti-YB-1 shRNA expression system was established.

Establishment of tetracycline repressor expressing cancer cells

The classical multidrug-resistant gastrointestinal cancer cell lines EPG85-257RDB and EPP85-181RDB as well as their parental, drug-sensitive counterparts, EPG85-257P and EPP85-181P, were used to generate tetracycline repressor expressing sublines, EPG85-257P/tetR, EPP85-181P/tetR, EPG85-257RDB/tetR, and EPP85-181RDB/tetR, by stable transfection using the tetracycline repressor encoding vector pcDNA6/TR. For selection of clones used for transfection of shRNA encoding tetracycline-depending expression vectors the efficacy of the tetracycline-inducible expression system was analyzed in the tetracycline repressor synthesizing cells using the luciferase reporter gene encoding plasmid pcDNA4TOluc [23]. Clones used

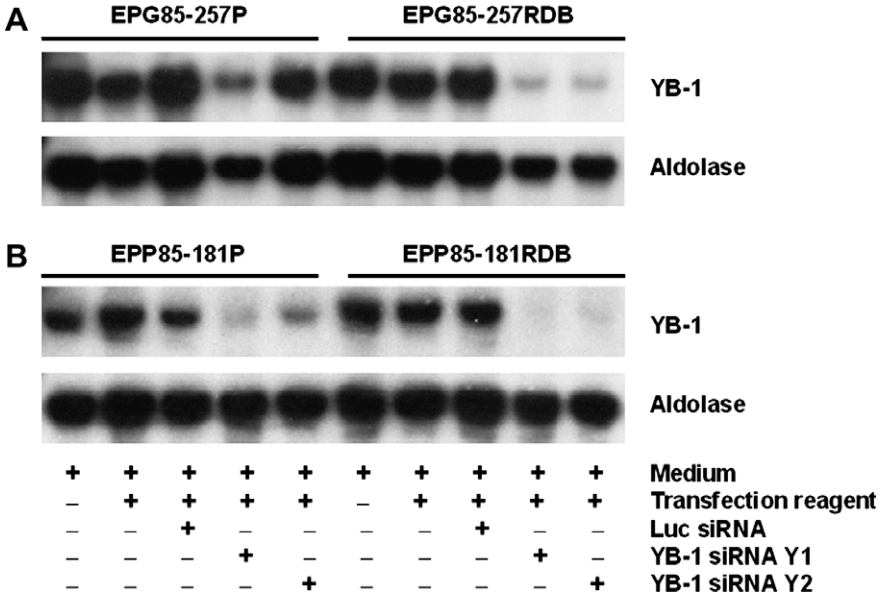

Fig. 1. SiRNA-mediated decrease of YB-1 mRNA expression after three days of siRNA treatment. **(A)** Northern blot analysis of the parental, drug-sensitive gastric cancer cell line EPG85-257P and its multidrug-resistant counterpart EPG85-257RDB. **(B)** Northern blot analysis of the parental, drug-sensitive pancreatic carcinoma cell line EPP85-181P and its multidrug-resistant derivative EPP85-181RDB. As loading control, the blotting membranes were stripped and reprobed using an aldolase-specific cDNA. Medium, untreated; Transfection reagent, Oligofectamine without siRNA molecules; Luc siRNA, control siRNA directed against luciferase; YB-1 siRNA Y1, YB-1-specific siRNA 1; YB-1 siRNA Y2, YB-1-specific siRNA 2.

Table 1
Anthracycline resistance of anti-YB-1 siRNA/shRNA-treated cancer cells

Cell variant	RNAi-triggering construct	Tetracycline	Daunorubicin IC_{50} value (μM)
Gastric carcinoma cells			
EPG85-257P	Medium		0.012
EPG85-257RDB	Medium		12.1
EPG85-257P	Transfection reagent		0.012
EPG85-257RDB	Transfection reagent		11.5
EPG85-257P	Luc siRNA		0.011
EPG85-257RDB	Luc siRNA		13.0
EPG85-257P	YB-1 siRNA Y1		0.011
EPG85-257RDB	YB-1 siRNA Y1		12.7
EPG85-257P	YB-1 siRNA Y2		0.012
EPG85-257RDB	YB-1 siRNA Y2		13.7
EPG85-257RDB/tetR	Medium	−	14.5
EPG85-257RDB/tetR	Medium	+	15.0
EPG85-257RDB/tetR	Luc shRNA	−	11.8
EPG85-257RDB/tetR	Luc shRNA	+	12.0
EPG85-257RDB/tetR	YB-1 shRNA Y1	−	13.8
EPG85-257RDB/tetR	YB-1 shRNA Y1	+	12.5
EPG85-257RDB/tetR	YB-1 shRNA Y2	−	12.8
EPG85-257RDB/tetR	YB-1 shRNA Y2	+	12.5
Pancreatic carcinoma cells			
EPP85-181P	Medium		0.036
EPP85-181RDB	Medium		20.1
EPP85-181P	Transfection reagent		0.036
EPP85-181RDB	Transfection reagent		25.9
EPP85-181P	Luc siRNA		0.034
EPP85-181RDB	Luc siRNA		23.8
EPP85-181P	YB-1 siRNA Y1		0.030
EPP85-181RDB	YB-1 siRNA Y1		22.3
EPP85-181P	YB-1 siRNA Y2		0.032
EPP85-181RDB	YB-1 siRNA Y2		19.2
EPP85-181RDB/tetR	Medium	−	18.7
EPP85-181RDB/tetR	Medium	+	18.1
EPP85-181RDB/tetR	Luc shRNA	−	20.6
EPP85-181RDB/tetR	Luc shRNA	+	19.8
EPP85-181RDB/tetR	YB-1 shRNA Y1	−	22.3
EPP85-181RDB/tetR	YB-1 shRNA Y1	+	24.7
EPP85-181RDB/tetR	YB-1 shRNA Y2	−	23.4
EPP85-181RDB/tetR	YB-1 shRNA Y2	+	26.2

for the transfection procedure showed 80–100-fold increased relative luciferase activity following tetracycline exposure.

Establishment of tetracycline-dependent anti-YB-1 shRNA expressing cancer cells

The tetracycline repressor expressing cell variants, EPG85-257P/tetR, EPP85-181P/tetR, EPG85-257RDB/tetR, and EPP85-181RDB/tetR, were used for establishment of tetracycline-inducible anti-YB-1 shRNA expressing cell variants EPG85-257P/tetR/YB-1 shRNA Y1, EPG85-257P/tetR/YB-1 shRNA Y2, EPP85-181P/tetR/YB-1 shRNA Y1, EPP85-181P/tetR/YB-1 shRNA Y2, EPG85-257RDB/tetR/YB-1 shRNA Y1, EPG85-257RDB/tetR/YB-1 shRNA Y2, EPP85-181RDB/tetR/YB-1 shRNA Y1, and EPP85-181RDB/tetR/YB-1 shRNA Y2, as well as control cell lines expressing anti-luciferase shRNAs, EPG85-257P/tetR/Luc shRNA, EPP85-181P/tetR Luc shRNA, EPG85-257RDB/tetR Luc shRNA, and EPP85-181RDB/tetR Luc shRNA.

Modulation of YB-1 mRNA and protein expression by tetracycline-dependent anti-YB-1 shRNA expression

Northern blot and Western blot experiments demonstrated that tetracycline treatment of stable anti-YB-1 shRNA expression vector transfected cell lines resulted in a complete disappearance of YB-1-specific bands in all cell lines, EPG85-257P/tetR/YB1 shRNA Y1, EPG85-257P/tetR/YB-1 shRNA Y2, EPP85-181P/tetR/YB-1 shRNA Y1, EPP85-181P/tetR/YB-1 shRNA Y2, EPG85-257RDB/tetR/YB-1 shRNA Y1, EPG85-257RDB/tetR/YB-1 shRNA Y2, EPP85-181RDB/tetR/YB-1 shRNA Y1, and EPP85-181RDB/tetR/YB-1 shRNA Y2. Figs. 2A and B demonstrate these effects exemplarily

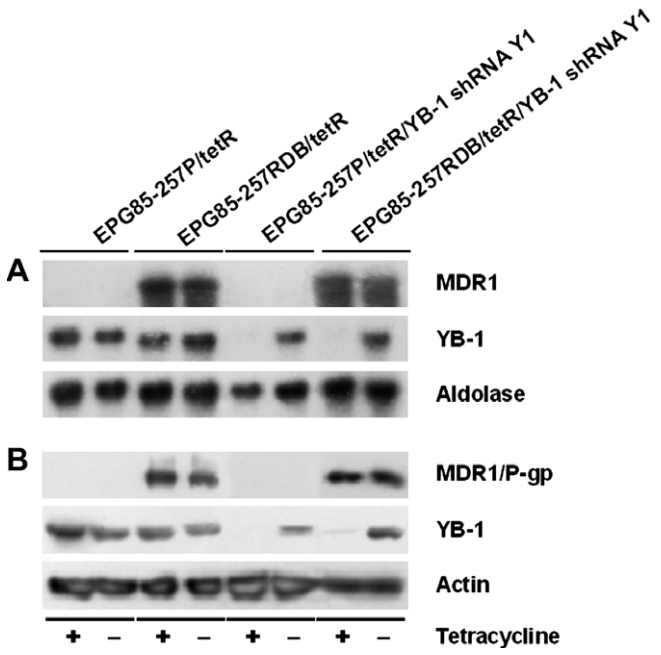

Fig. 2. MDR1 mRNA and MDR1/P-gp protein expression as well as shRNA-mediated decrease of YB-1 mRNA and protein expression in human gastric carcinoma cells. **(A)** Northern blot analysis depicting silencing of YB-1 mRNA expression following treatment with tetracycline and the expression of the MDR1 mRNA. As loading control, the Northern blot membranes were probed using an aldolase-specific cDNA. **(B)** Western blot analysis of YB-1 expression as result of tetracycline treatment in gastric carcinoma cells. As control for equivalent protein loading, the filter was incubated with a mouse mAb directed against actin. EPG85-257P/tetR, parental EPG85-257P cells expressing the tetracycline repressor; EPG85-257RDB/tetR, multidrug-resistant EPG85-257RDB cells expressing the tetracycline repressor; EPG85-257P/tetR/YB-1 shRNA Y1, EPG85-257P/tetR cells containing the anti-YB-1 shRNA Y1 encoding tetracycline-depending expression vector; EPG85-257RDB/tetR/YB-1 shRNA Y1, EPG85-257RDB/tetR cells containing the anti-YB-1 shRNA Y1 encoding tetracycline-depending expression vector.

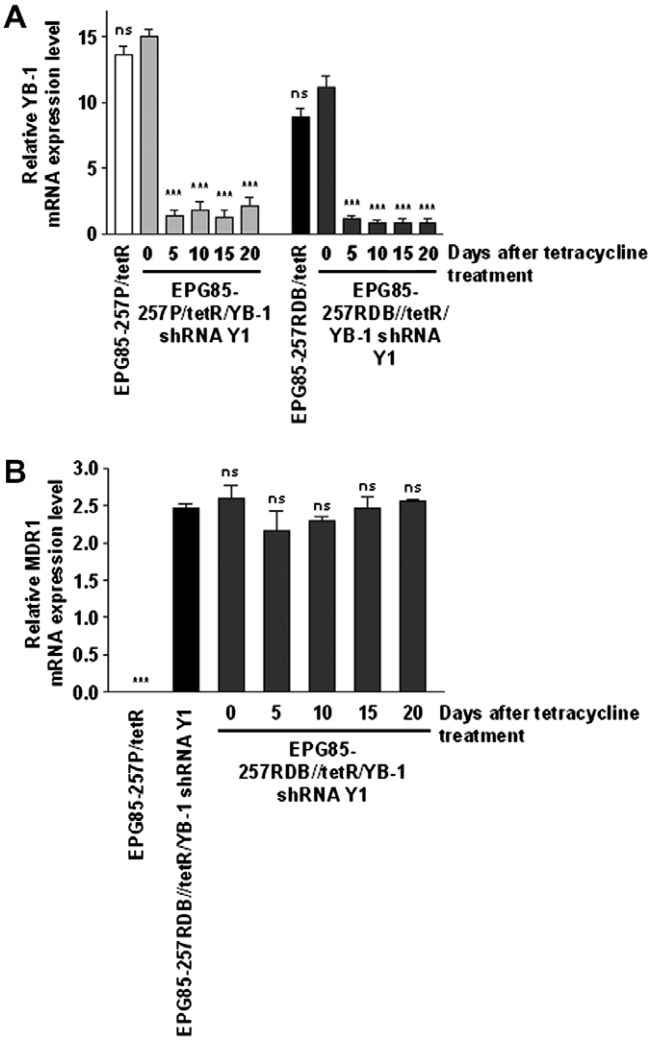

Fig. 3. Long term analysis of time-depending **(A)** YB-1 mRNA and **(B)** MDR1 mRNA expression by quantitative real-time RT-PCR in human gastric carcinoma cells following tetracycline treatment. Relative YB-1 and MDR1 mRNA expression levels were normalized against aldolase mRNA expression levels. EPG85-257P/tetR, parental EPG85-257P cells expressing the tetracycline repressor; EPG85-257RDB/tetR, multidrug-resistant EPG85-257RDB cells expressing the tetracycline repressor; EPG85-257RDB/tetR/YB-1 shRNA Y1, EPG85-257RDB/tetR cells containing the anti-YB-1 shRNA Y1 encoding tetracycline-depending expression vector (ns, no significant difference from the untreated cell line; $^{***}p < 0.001$).

for the multidrug-resistant gastric carcinoma cell variant EPG85-257RDB/tetR/YB-1 shRNA Y1 and its drug-sensitive counterpart EPG85-57P/tetR/YB-1 shRNA Y1.

Quantitative real-time RT-PCR demonstrated that the shRNA-mediated YB-1 mRNA down regulation was stable for at least 20 days (Fig. 3A). An induced YB-1 silencing longer than 15 days led to a significant increase in the percentage of apoptotic cells with characteristic cell morphology (data not shown).

Influence of tetracycline-dependent YB-1 down regulation on MDR1/P-gp mRNA and protein expression

Tetracycline-dependent down regulation of YB-1 had no effect on the MDR1 mRNA expression (Fig. 2A) or MDR1/P-gp protein expression. Quantitative real-time RT-PCR showed that for at least 20 days no influence of YB-1 down regulation on the MDR1 expression could be observed (Fig. 3B). Therewith, it could be excluded that potential biological effects of YB-1 on the MDR1 gene activity would take place delayed.

In line with the information obtained using anti-YB-1 siRNAs these data confirm that MDR1 expression is completely independent from YB-1.

Anthracycline sensitivity of anti-YB-1 shRNA expressing cancer cells

As expected from the previous experiments using anti-YB-1 siRNAs, the shRNA-mediated down regulation of YB-1 has no effects on the sensitivity against anthracyclines (Table 1). Thus, the complete independence of the MDR1-mediated multidrug-resistant phenotype was confirmed. A previous study applied RNAi technology for down regula-

tion of YB-1 in MDR1-positive cells, which includes ambiguous data [12]. In that approach, naked T7 RNA polymerase-synthesized anti-YB-1 shRNAs were used to transfect the cancer cell lines HCT116, MCF-7/Adr, and mS-0.5. In HCT116 and MCF-7/Adr cells no down regulation of the YB-1 mRNA could be observed, in the melanoma cell line mS-0.5 merely a slight effect was barely detected. According to this observation, no effects on the MDR1 mRNA expression could be found in HCT116 and MCF-7/Adr cells, as well as negligible effects in mS-0.5 cells. These effects are not convincing, because the applied RT-PCR technique is not quantitative. Thus, also that data support the conclusion that endogenous MDR1 gene expression is independent from YB-1.

Conclusions

It was demonstrated that the RNAi technology is effective in modulation of YB-1 expression in different multidrug-resistant cancer cells and their parental, drug-sensitive counterparts. Therewith, the data demonstrate the utility of the analyzed siRNAs and tetracycline-depending shRNA expression vectors as powerful laboratory tools for investigation of YB-1-mediated biological effects. This is of particular interest, as YB-1 is involved in the transcriptional and translational control of many biological processes, including cell proliferation [28]. The data show that there was no involvement of YB-1 in the regulation of the endogenous MDR1 gene in MDR cells. This effect is in line with the fact that YB-1 does not interact with double-stranded oligonucleotides containing the MDR1 CCAAT box sequence indicating that YB-1 has no transcriptional activity for MDR1 gene expression [5]. Although a number of studies have linked the expression or nuclear localization of YB-1 with increased MDR1 expression [6,8–11], our data support the conclusions by others [2,5] that a direct involvement of YB-1 in MDR1 transcription appears unlikely. Whether YB-1 is activated in parallel with MDR1/P-gp as part of stress response or whether there are alternative non-transcriptional roles of YB-1, e.g. maintenance of MDR1/P-gp activity has to be clarified. However, the data show that YB-1 does not appear as a potential target for therapeutically overcoming of MDR.

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