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ORIGINAL ARTICLE

MicroRNA-143 replenishment re-sensitizes colorectal cancer cells harboring mutant, but not wild-type, KRAS to paclitaxel treatment

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Abstract Colorectal cancer (CRC) global incidence is one of the highest among cancers. The *KRAS* gene has been shown as a robust biomarker for poor prognosis and drug resistance. MicroRNA-143 (*miR-143*) and *let-7* are families of tumor suppressor microRNAs that are often downregulated in CRC, especially with coexistent *KRAS* mutations. In order to evaluate if *miR-143* and/or *let-7b* replenishment would re-sensitize CRC cells to paclitaxel treatment, we investigated in effect of *miR-143* and *let-7b* replenishments on sensitivity to paclitaxel treatment in *KRAS* mutant *LoVo* and wild-type SW48 CRC cell lines. Our results showed that *miR-143*, but not *let-7b*, increased sensitization of *KRAS* mutant tumor cells to paclitaxel. Furthermore, transfection of *miR-143*, but not *let-7b*, mimic negatively regulated the expression of mutant but not wild-type *KRAS*. Combination of *miR-143* mimic and paclitaxel induced the onset of apoptosis, and reverted in vitro metastatic properties (migration and invasion) in *KRAS* mutant tumor cells. *MiR-143* thus can be used as a chemosensitizer for the treatment of *KRAS* mutant tumors and warrants further investigations in in vitro and pre-clinical in vivo models.

Keywords Colorectal cancer · KRAS · miR-143 · let-7b

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Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, accounting for approximately 9 % of cancer-related deaths [1], mostly due to metastasis that is a result of resistance to immuno/chemotherapy [2]. Most CRC patients are diagnosed with localized disease amenable to curative surgical resection [3]. However, approximately 20 % of patients are diagnosed with distant metastases [3], highlighting the need for more advanced therapeutic regimens.

Neoadjuvant chemotherapy has assumed increasing importance in the treatment of CRC, replacing surgery as the sole therapy of choice [3]. The *KRAS* gene has been shown to have inverse prognostic power in predicting whether a CRC patient will respond to such neoadjuvant therapies [4]. Close to 35–45 % of patients with CRC has mutant *KRAS* [5–11]. Ninety-five percent of *KRAS* mutation occur in exons 12 and 13, with the former alone contributing to 80 % of detected mutations [4]. It has been observed that patients harboring mutant *KRAS* have a tendency to develop resistance to neoadjuvant chemotherapy and hence suffer from poor response rate to chemotherapy.

MicroRNAs (miRNAs), small noncoding endogenously produced RNAs, can function as both tumor suppressors and oncogenes by either causing target mRNA degradation or inhibiting their translation [12, 13]. *Mir-143* has been shown to target *KRAS* in both CRC [14] and prostate cancer [15]. The human *let-7* family of tumor suppressor miRNAs also targets *KRAS* [16] and has been shown to be frequently lost in human cancers [17, 18]. Even though replenishment of *let-7* and *miR-143* seem to be a viable treatment strategy, *let-7* replenishment failed to arrest proliferation and induce cell death in *KRAS* mutant tumor cells, severely limiting its clinical use [19, 20]. Thus, the objective of the current study was to determine if *let-7b* or *miR-143* replenishment would function as a

chemosensitizer for conventional chemotherapeutic agent like paclitaxel (PTX) in *KRAS* mutant and wild-type tumor cells.

Materials and methods

Cell culture

Human colon cancer cell lines SW48 (wild-type *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, and *TP53*) and LoVo (mutant *KRAS*-G13D, A14V, wild-type *BRAF*, *PIK3CA*, *PTEN*, and *TP53*) [21] were obtained from the ATCC (Manassas, VA, USA) and maintained at 37 °C in a CO_2 incubator in Dulbecco's modified Eagle's media (DMEM) containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin. This article does not contain any studies with human participants or animals performed by any of the authors.

Cell transfection and treatments

Hsa-miR-143 mimic, *let-7b-5p* mimic, and the non-targeting (scramble oligonucleotides) control (SCR) (Life Technologies, Beijing, China). Cells (4×10^4) were transiently transfected with 50 nM of the mimics or scramble control using Lipofectamine LTX (Life Technologies) as per the manufacturer's guidelines. Twelve hours after transfection, the cells were subjected to treatment with indicated concentrations of PTX (Sigma-Aldrich, Beijing, China) for 36 h.

Analysis of combination effects

CompuSyn software (ComboSyn, Paramus, NJ) [21] was used to calculate the combination index (CI) between PTX and *let-7b* or *miR-143*. Synergistic, additive, and antagonistic effects were defined as CI<0.9, CI=0.9–1.1, or CI>1.1, respectively [22].

RNA and miRNA extraction and qRT-PCR

TRIzol reagent was used to isolate total RNA. First-strand cDNA was synthesized using the First Strand cDNA synthesis Kit (Life Technology), which was then used for qRT-PCR using TaqMan Gene Expression probes (Life Technology). *TBP* (TaqMan Assay ID Hs00427620_m1) was used as an internal control for assessing *KRAS* (TaqMan Assay ID Hs00364284_g1) transcript level. Data was normalized to *TBP* expression and analyzed by the $-\Delta\Delta$Ct method. MiRNA from tissues and cells was extracted using the mirVana miRNA isolation kit (Life Technology), and TaqMan miRNA assays (Life Technology (TaqMan Assay IDs 002146, 002619, and 001093, respectively) were used to quantify the expression levels of *hsa-miR-143*, *let-7b*, and *RNU6B*. Data was normalized to *RNU6B* expression and analyzed by the $-\Delta\Delta$Ct method.

Preparation of whole cell lysates and immunoblot analysis

Cells were lysed in lysis buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % NP-40 and 5 % glycerol) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, USA). Lysates were resolved by SDS-PAGE and probed with KRAS and PARP antibody (Abcam, Cambridge, USA). The blot was probed for β-actin (Cell Signaling, Danvers, USA) to confirm equal loading.

Cell viability assay

Cell proliferation was quantitated using a mitochondrial colorimetric assay (MTT assay, Sigma-Aldrich, St. Louis, MO) as per the manufacturer's recommendations. The absorbance was measured at 570 nm. The results, expressed in terms of relative optical density (OD), as mean±standard deviation.

Apoptosis assay

Apoptosis was assessed by using the Annexin V and PI staining kit (BD Biosciences, San Jose, CA, USA) as per the manufacturer's recommendations.

Cell proliferation assay

Apoptosis was assessed by using the CFSE staining kit (LifeTechnology, Carlsbad, CA, USA) as per the manufacturer's recommendations.

In vitro transwell migration and invasion assays

For in vitro migration assays, indicated cells treated with mitomycin C were serum starved, trypsinized, and introduced into the upper chamber (1×10^5) of the transwell (8-μm pore size). Migration and invasion analysis was done using Culturex 96-well cell migration and Culturex 96-well BME cell invasion assay kits (R&D Systems, Beijing, China), respectively. Data obtained from both sets of experiments were used to analyze percent migration and invasion and were expressed as mean±standard error of mean (SEM).

Statistical analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corporation, NY). A $P<0.05$ was considered statistically significant.

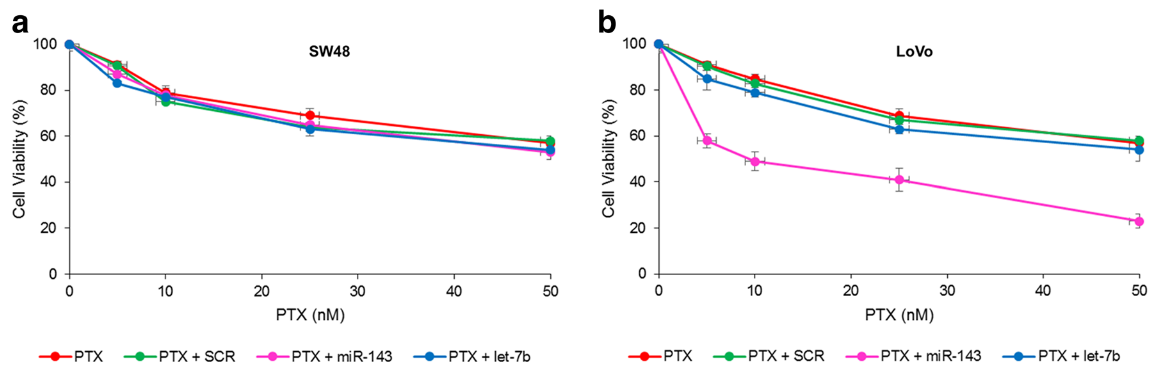

Fig. 1 *MiR-143*, but not *let-7b*, repletion chemosensitizes *KRAS* mutant, but not wild-type colon cancer tumor cells. Colon cancer cells SW48 (*KRAS* wild-type) (**a**) and LoVo (*KRAS* mutant) (**b**) were transfected with either 50 nM *miR-143* mimic, *let-7b* mimic, or a scramble control (SCR) for 12 h. The cells were then treated with paclitaxel (PTX, 0.1–50 nM) for 36 h. Cell viability was assessed by the crystal violet assay. Each experiment was carried out at least three times. Data represent mean ±SEM

Results

MiR-143, but not *let-7b*, replenishment selectively enhances the chemosensitivity of *KRAS* mutant tumor cells

To determine the therapeutic potential of *miR-143* and *let-7b* as a chemosensitizer, we evaluated the effect of *miR-143* and *let-7b* replenishment on the cytotoxicity of PTX in *KRAS* wild-type SW48 (Fig. 1a) and mutant LoVo (Fig. 1b) cells. PTX is routinely used for treatment of solid tumors, and hence, we chose the same. Transfection of *miR-143* mimic significantly chemosensitized LoVo cells, which harbor *KRAS* mutation, but did not affected *KRAS* wild-type SW48 cells (Fig. 1a). No effect with *let-7b* mimic was seen in either cell line (Fig. 1).

MiR-143 mimic decreased the IC_{50} of PTX from 23±3 to 6 ±1 nM in LoVo cells, while the reduction was less than 1 % in *KRAS* wild-type SW48 cells (Fig. 2). As a negative control, the scramble control minimally influenced the cytotoxicity of PTX in either cell line. To determine whether *miR-143*/PTX combination resulted in synergistic effect, the combination index was calculated from the proliferation data generated in each cell line. This analysis showed that the combination indices of *miR-143*/PTX scored 0.53 in *KRAS* mutant LoVo cells compared to 1.09 in SW48 cells, signifying a synergistic response in cells containing mutant *KRAS* (Fig. 2). *Let-7b* had a combination index >1 in both cell lines (Fig. 2). Cumulatively, these results indicate that *miR-143* replenishment selectively sensitizes *KRAS* mutant cells to the cytotoxicity of PTX.

MiR-143 selectively downregulates mutant *KRAS* expression

To understand the differential effect of *miR-143* and *let-7b* on the wild-type versus mutant *KRAS* tumor cells in response to PTX, we first compared the endogenous levels of *miR-143* and *let-7b* and *KRAS* in these two cell lines. The expression of *miR-143* was 25-fold higher in SW48 compared to LoVo cells (Fig. 3a). Conversely, *KRAS* mRNA (Fig. 3b) and protein (Fig. 3c) expression was significantly higher in LoVo cells compared to SW48 cells. Both SW48 and LoVo cells expressed comparable levels of *let-7b*. Next, we examined KRAS expression in response to *miR-143*/PTX treatment. Transfection of *miR-143* mimic restored intracellular *miR-143* to a similar level in both cell lines regardless of *KRAS* mutational status or drug exposure (Fig. 4a). We found that irrespective of PTX exposure, transfection of *miR-143* mimic markedly reduced *KRAS* mRNA expression in *KRAS* mutant LoVo cells (Fig. 4b), whereas the effect was insignificant in *KRAS* wild-type SW48 cells. These results are indicative of the fact that mutant *KRAS* is more susceptible to the negative modulation by *miR-143* than the wild-type *KRAS*.

Combination of *miR-143* with PTX induces apoptosis in *KRAS* mutant LoVo cells

Mutant *KRAS* constitutively activates MEK/ERK and PI3K/AKT signaling pathways, both of which are pivotal to the survival and proliferation of tumor cells [23, 24]. Thus, we next investigated if combination PTX and *miR-143* mimic

	IC_{50} of Paclitaxel				
	Scramble (nM)	hsa-miR-143 (nM)	CI_{miR_143}	Let-7b (nM)	CI_{let-7b}
SW48 (*KRAS* Wild Type)	25 ± 3	23 ± 3	1.09	23 ± 2	1.05
LoVo (*KRAS* G13D Mutant)	23 ± 2	6 ± 1	0.53	22 ± 1	1.03

CI, combination index. CI > 1.1 = antagonism; CI = 0.9 – 1.1 = additive; C < 0.9 - synergism

Fig. 2 Effect of *miR-143* or *let-7b* repletion on the cytotoxicity of paclitaxel in the *KRAS* mutant LoVo and wild-type SW48 cell lines. Each experiment was carried out at least three times

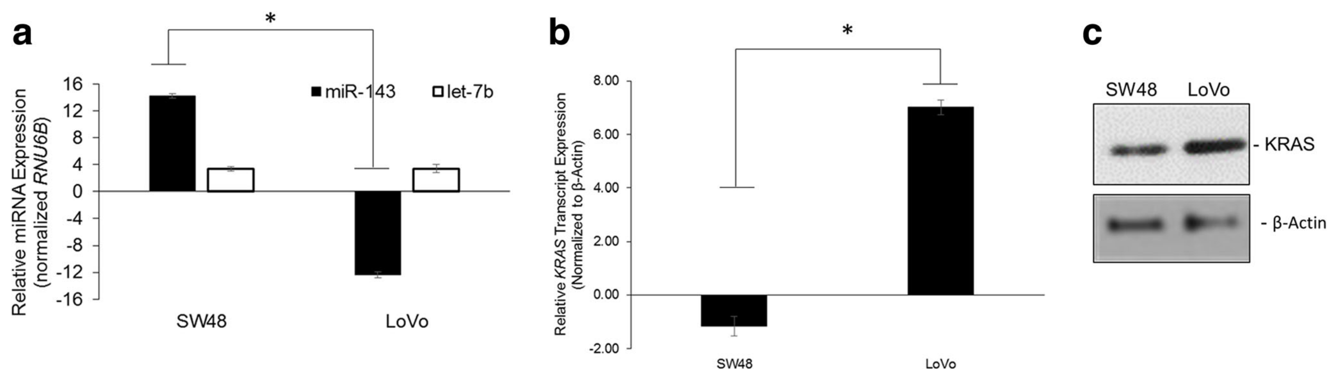

Fig. 3 Detection of steady state expression levels of *miR-143*, *let-7b*, and *KRAS* mRNA and protein in the *KRAS* mutant LoVo and wild-type SW48 cell lines. **a** The endogenous levels of *miR-143* and let-7b, normalized to *RNU6B* in SW48 and LoVo cell lines. The endogenous levels of *KRAS* mRNA normalized to β-actin (*ACTB*) expression (**b**), and KRAS protein (**c**) in SW48 and LoVo cells. The Western blot was re-probed with β-actin to serve as a loading control. Each experiment was carried out at least three times. Data represent the mean±SEM. $*P<0.05$

treatment induced apoptosis in the mutant *KRAS* harboring LoVo cells. Annexin V/PI-staining showed that combination of *miR-143* mimic with PTX decreased percent live cells by 70 % as compared to 40 % with either reagent alone (Fig. 5a, b). The apoptotic markers, PARP, were more strongly induced in the cells co-treated with *miR-143*/PTX than either agent alone (Fig. 5c). Furthermore, the induction of apoptosis was not accompanied by an inhibition of proliferation as determined by similar CFSE staining pattern in PTX and combined *miR-143* mimic and PTX treated LoVo cells over 72 h ($P=0.893$) (Fig. 5d).

Combination of *miR-143* with PTX markedly reduces migration and invasion of *KRAS* mutant tumor cells

LoVo cells are well characterized for their high motility [25], which is reflective of the metastatic nature of CRC. We next investigated the effect of *miR-143*/PTX combination on in vitro migration and invasion of LoVo cells as it will be a direct indicator of the potential of this combination therapy for in vivo usage. As shown in Fig. 6, treatment of PTX or *miR-143* alone caused 20–30 % reduction in both in vitro migration and invasion, whereas around 50–55 % decrease were observed when both *miR-143* and PTX were used ($P<0.05$).

Discussion

Restoration of *miR-143* alone or in combination with *miR-145* has been previously shown to reduce tumorigenic potential in pancreatic [26], bladder [27], and colorectal [28] cancers. However, there has been no study reported looking at the potential of *miR-143* replenishment as a sensitizer to reduce chemoresistance in cancer cells, inclusive of CRC. On the other hand, *let-7* family members can also attenuate chemoresistance [29–33]. We however did not observe any *let-7b*-mediated re-sensitization to drug-induced chemoresistance.

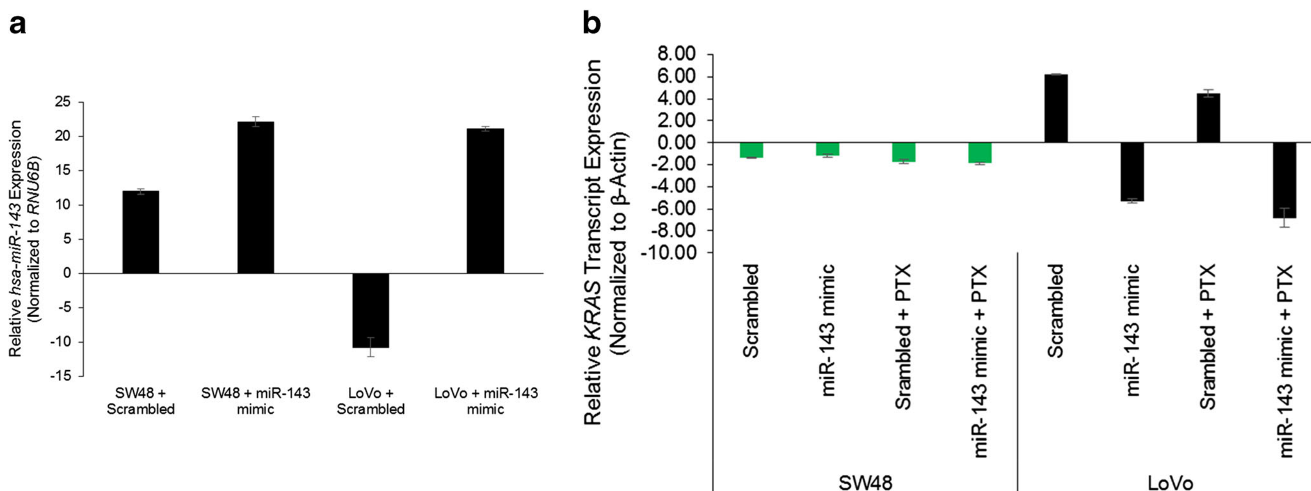

Fig. 4 *Mir-143* selectively downregulates mutant *KRAS* expression. The expression of *miR-143* (**a**) and *KRAS* mRNA (**b**) in the SW48 and LoVo cells transfected with scrambled control (SCR) or *miR-143* mimic alone or in combination with paclitaxel (PTX). Each experiment was carried out at least three times. Data represent the mean±SEM. $*P<0.05$

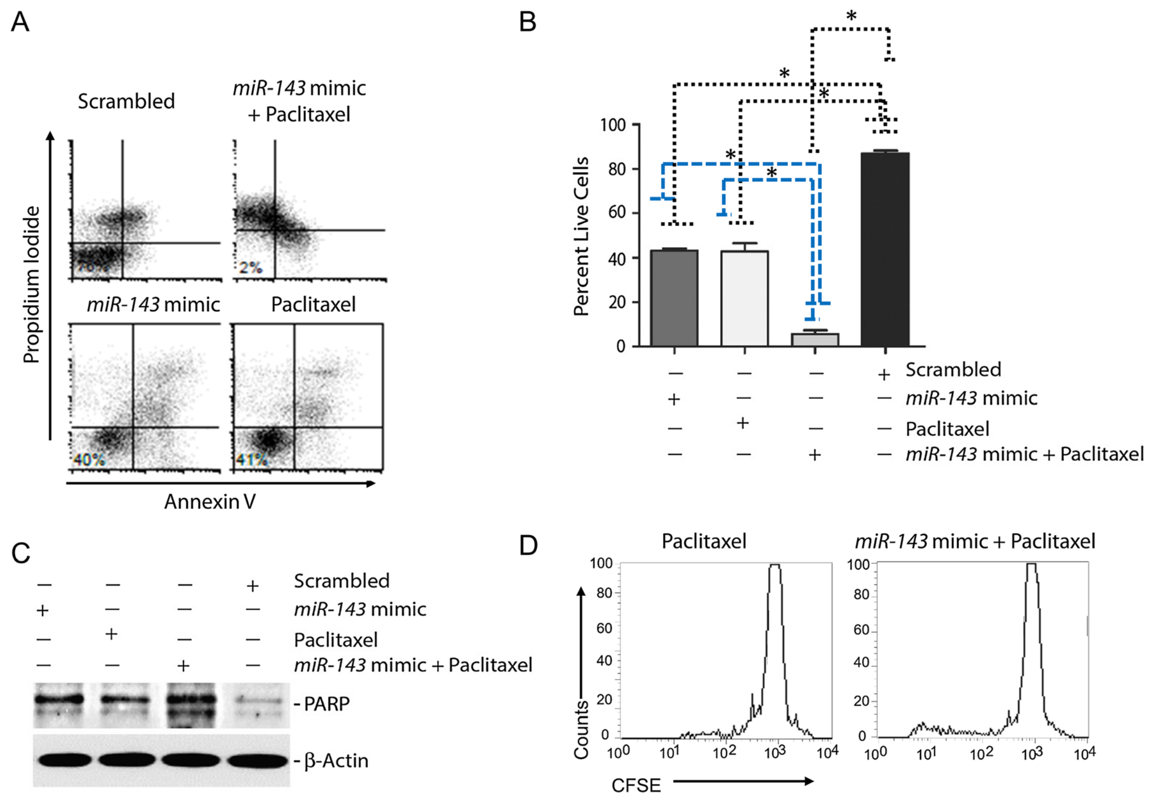

Fig. 5 *MiR-143* mimic and paclitaxel (PTX) combination blocks mutant *KRAS* signaling and induces apoptosis. The effect of scrambled control (SCR), *miR-143* mimic, PTX, or *miR-143* mimic+PTX on cell death in mutant *KRAS* LoVo cells. **a** The apoptotic cells were detected by flow cytometry using Annexin V-FITC and PI dual staining and Annexin V and PI double-negative cells were plotted as percent live cells. Data represent the mean±SEM. **P*<0.05. **b** The apoptotic protein marker (poly(ADP-ribose) polymerase (PARP)) was assessed by Western blotting. The blot was re-probed with β-actin to serve as a loading control. Each experiment was carried out at least three times

In the present study, our results show that *miR-143* can function as a chemosensitizer in drug-naive *KRAS* mutant cancer cells. Given that *miR-143* has cognate binding site in the *KRAS* mRNA's 3′-untranslated region (UTR), irrespective of any coding region mutation, it is surprising that transfection of *miR-143* mimic only had effect on diminishing the expression of mutant and not wild-type *KRAS* mRNA (Fig. 4b). This can perhaps be explained if suppression of *KRAS* expression by *miR-143* is dictated by the stoichiometry between *KRAS* mRNA and *miR-143* in tumor cells and not the binding capacity to the *KRAS* mRNA per se. In support of this hypothesis is the fact that compared to cells with wild-type

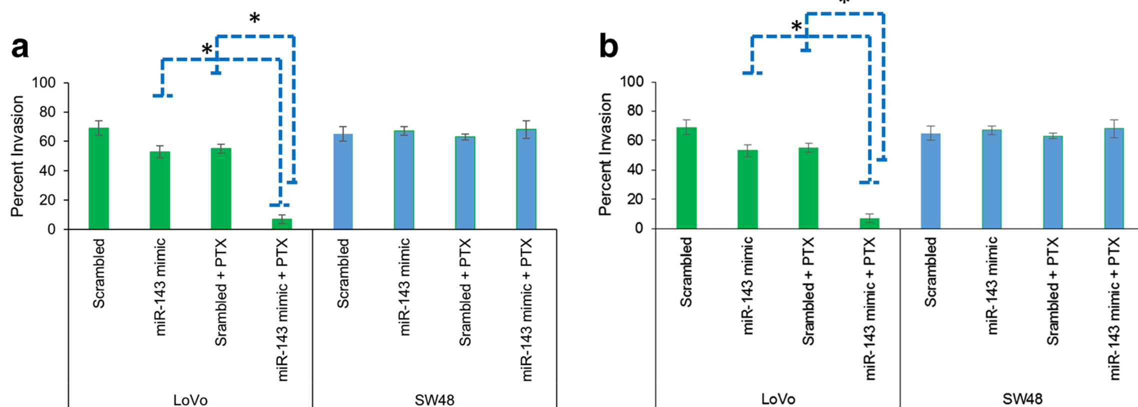

Fig. 6 *MiR-143* mimic and paclitaxel (PTX) combination inhibits migration and invasion of *KRAS* mutant LoVo, but not wild-type SW48 cells. **a** The effect of *miR-143* mimic or PTX alone and in combination on cell migration was evaluated in SW48 and LoVo cells for 8 h. **b** Matrigel invasion assay was conducted to evaluate the effect of *miR-143* mimic or PTX alone and in combination on the invasiveness of LoVo and SW48 cells. Each experiment was carried out at least three times. Data represent the mean±SEM. **P*<0.05

KRAS, cells harboring mutant *KRAS* had higher steady-state expression of *KRAS* mRNA and protein coupled with decreased *miR-143* expression (Fig. 3). Hence, in *KRAS* mutant cells, the low expression of *miR-143* is rate limiting, which will also explain the significant suppression of *KRAS* mRNA level induced by *miR-143* mimic. The high levels of *miR-143* in cells with wild-type *KRAS* will thus mean optimal targeting of the *KRAS* mRNA, which is not further potentiated by *miR-143* mimic. Of note, though that suppression of mutant *KRAS* by *miR-143* alone could not attenuate cell proliferation or induce cell death, which corroborates previous findings that RNAi-mediated downregulation of mutant *KRAS* has limited antitumorigenic potential [33]. The later can be explained by the fact that RAS-independent signaling pathways would still be operant when mutant *KRAS* are suppressed by RNAi.

As shown in Fig. 4a, transfection of *miR-143* mimic replenished the low endogenous levels of *miR-143* in LoVo cells to the abundance level in parental or *miR-143* mimic transfected SW480 cells. Now, if our hypothesis is correct, it would mean that *miR-143* being at a stoichiometric excess post-transfection of the mimic in LoVo cells should be able to successfully target *KRAS* transcript and suppress its expression. Now, referring to Fig. 4b showed us whereas scrambled transfected LoVo cells (low *miR-143* stoichiometry) had high *KRAS* expression, *miR-143* mimic transfected LoVo cells (high *miR-143* stoichiometry) had low KRAS expression. In fact, the mimic transfected LoVo cells suppressed *KRAS* more than in the SW480 cells. This suppression was even more potentiated by co-treatment with paclitaxel. It will be potentially interesting to evaluate of the reverse experiment using *miR-143* antagomir in SW480 cells would increase *KRAS* expression in a similar fashion.

In conclusion, our results show that *miR-143* can potentially sensitize tumor cells harboring mutant *KRAS* to treatment with PTX, which has obvious clinical benefits for treating tumors with mutant *KRAS*. Given that the response rate to PTX therapy alone is normally less than 30 %, use of *miR-143* mimic should significantly improve the therapeutic outcome and mitigate drug resistance, which calls for more clinical trials involving the same hypothesis.

Compliance with ethical standards

Conflicts of interest None

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