Oncogene (2012), 1–8
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www.nature.com/onc

ORIGINAL ARTICLE

Analysis of the combined action of miR-143 and miR-145 on oncogenic pathways in colorectal cancer cells reveals a coordinate program of gene repression

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MicroRNAs (miRNAs) from the gene cluster miR-143–145 are diminished in cells of colorectal tumor origin when compared with normal colon epithelia. Until now, no report has addressed the coordinate action of these miRNAs in colorectal cancer (CRC). In this study, we performed a comprehensive molecular and functional analysis of the miRNA cluster regulatory network. First, we evaluated proliferation, migration, anchorage-independent growth and chemoresistance in the colon tumor cell lines after miR-143 and miR-145 restoration. Then, we assessed the contribution of single genes targeted by miR-143 and miR-145 by reinforcing their expression and checking functional recovery. Restoring miR-143 and miR-145 in colon cancer cells decreases proliferation, migration and chemoresistance. We identified cluster of differentiation 44 (CD44), Kruppel-like factor 5 (KLF5), Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) and v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) as proteins targeted by miR-143 and miR-145. Their re-expression can partially revert a decrease in transformation properties caused by the overexpression of miR-143 and miR-145. In addition, we determined a set of mRNAs that are diminished after reinforcing miR-143 and miR-145 expression. The whole transcriptome analysis ascertained that downregulated transcripts are enriched in predicted target genes in a statistically significant manner. A number of additional genes, whose expression decreases as a direct or indirect consequence of miR-143 and miR-145, reveals a complex regulatory network that affects cell signaling pathways involved in transformation. In conclusion, we identified a coordinated program of gene repression by miR-143 and miR-145, in CRC, where either of the two miRNAs share a target transcript, or where the target transcripts share a common signaling pathway. Major mediators of the oncosuppression by miR-143 and miR-145 are genes belonging to the growth factor receptor–mitogen-activated protein kinase network and to the p53 signaling pathway.

Oncogene advance online publication, 5 November 2012; doi:10.1038/onc.2012.495

Keywords: RAS/MAPK; signaling pathway; miRNA; microarray; colorectal cancer

INTRODUCTION

The miR-143 and miR-145 genes are closely located in a 1.6 kb region on chromosome 5q33.1. These transcripts frequently display a coordinated expression profile suggesting they originate from the same precursor.[1,2] MiR-143 and miR-145 levels are decreased in diverse epithelial tumors such as those arising from the lung[3] and breast.[4] This suggests that a frequent oncogenic lesion, common to these neoplasms, might be implicated. One such lesion is the TP53 gene loss-of-function. It has recently been demonstrated that activation of the p53 pathway increases the miR-143 and miR-145 levels through a transcriptional mechanism.[5,6] Another mechanism that affects miR-143 and miR-145 levels depends on the mitogen-activated protein kinase (MAPK) cascade activity, with RREB1 being the terminal effector for the transcriptional repression of the cluster.[7] It is therefore of particular interest to consider microRNAs (miRNAs) as mediators of the actions of mutated oncogenes on cell behavior. Despite accumulating evidence on concomitant miR-143 and miR-145 downregulation in colon and gastric cancer, their function as players in a coordinated scheme of target gene regulation has only been studied in other biological contexts.[1,8,9]

Although the effect of miR-143 has been implicated in KRAS downmodulation in colorectal cancer (CRC), c-myc and insulin receptor substrate-1 (IRS-1) targeting may mediate the antiproliferative effect of miR-145.[6,10] This evidence was obtained from experiments of single miRNA overexpression and fit convincingly with the notion that the growth factor receptor (GFR)–RAS–MAPK axis and constitutive Wnt pathway activation, resulting in c-Myc induction, have a crucial role in CRC tumorigenesis. Hence, their targeting by miR-143 and miR-145 results in an adverse effect on the viability of the CRC-derived cell. Other miR-143 targets, such as DNA methyltransferase 3A (DNMT3A)[11] and extracellular signal-regulated kinase-5,[12] have been proposed to be mediators of an antiproliferative effect, but rarely a phenotypic rescue in these studies was attempted by transgenic expression of the targeted gene along with the miRNA that regulates the endogenous copy.

We have identified KLF5 and BRAF as novel miR-143 and miR-145 targets in CRC cells. Notably, KLF5 is required for KRAS-mediated transformation of normal colonic epithelium,[13] BRAF lies downstream of the already known target KRAS and is frequently mutated in CRC. The role of these target genes within the CRC framework has been refined by restoring their individual expression in a miR-143 and miR-145 overexpression background, assaying the consequences at the level of cell proliferation, and migration. Other than acting as players in the RAS–MAPK cascade, we found that CD44, a membrane

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Received 17 January 2012; revised 10 September 2012; accepted 13 September 2012

glycoprotein that mediates the transduction of survival signals and that is downregulated at the transcriptional level on p53 restoration,[14] is a directly repressed target for miR-143 and miR-145 in CRC cells. This highlights the focal impact of p53 in the regulation of CD44 by multiple mechanisms.

Our results, obtained both *in vitro* and in xenograft experiments, support the hypothesis that all the target genes we investigated are mediators of the miR-143–145 cluster oncosuppressor properties. However, we found that none of them is individually capable to fully restore the phenotypical properties of CRC cells that are affected by miR-143 and miR-145 expression. This led us to assess the overall transcriptome changes on enforcing miR-143 and miR-145 expression in a CRC-derived cell line, observing the modulation of transcripts belonging to additional pathways, which have a relevant role in tumorigenesis, such as cell cycle regulation machinery.

RESULTS

MiR-143–145 cluster expression is diminished in human CRC cell lines

Given that the downmodulation of miR-143 and miR-145 has been documented extensively in tumor tissues when compared with normal tissue, we assessed the extent of their decrease in CRC using normal colonic epithelium as a reference. Both miRNAs are detectable at low levels in the three CRC lines we examined, and their levels in normal tissue are up to three logs more abundant (Figure 1a).

Setting up a system for miR-143 and miR-145 expression, we considered that if endowed with oncosuppressor functions, they would induce a counter-selection when constitutively overexpressed in CRC cell lines. We overcame this problem by adopting the tet repressor technology,[15] an inducible system for gene expression allowing a rapid and regulatable transgene induction. HT-29, SW480 and HCT-116 cell lines were engineered to express high levels of red fluorescent protein (RFP), miR-143 and miR-145 on induction with doxycycline (see Materials and methods section). Infected cells were induced, RFP-positive cells were flow sorted and the induction of miR-143 and miR-145 was confirmed in all the cell lines (Figure 1b). We observed a maximum increase in HCT-116 cells, and therefore chose the derivatives HCT-LTR143-5, with inducible expression of RFP and miR-143 and miR-145, and HCT-LTR, with inducible expression of RFP, as an experimental model for the functional assays. Under continuous doxycycline induction, miR-143 and miR-145, as well as RFP expression, remained high over time (Supplementary Figure 1).

MiR-143–145 cluster expression inhibits oncogenic properties of CRC cell lines

Inducible expression of miR-143 and miR-145 had an inhibitory effect on the HCT-116 cell line growth properties (Figure 2a), with a stable decrease in growth rate. We then examined whether miR-143 and miR-145 expression could alter additional malignant features of HCT-116, such as migration. The motility of HCT-116 after miR-143 and miR-145 induction was examined using the transwell migration assay. We observed a significant reduction in the migration capabilities of HCT-LTR143-5 (Figure 2b), at an extent, which could not be attributed merely to the already mentioned decrease in the growth kinetics, given the short duration of the assay.

Cells engineered in the same way as in the migration assay were used to assess whether reinforced expression of miR-143 and miR-145 could influence the ability to grow in an anchorage-independent manner. To do this, doxycycline-induced cells were plated in soft agar and allowed to grow for 2 weeks. HCT-LTR143-5 cells formed fewer colonies as compared with HCT-LTR cells (Figure 2c). Thus, miR-143 and miR-145 expression resulted in considerable inhibition of proliferation, migration and colony formation of HCT-116, suggesting that these coexpressed miRNAs can have a pivotal role in CRC oncosuppression.

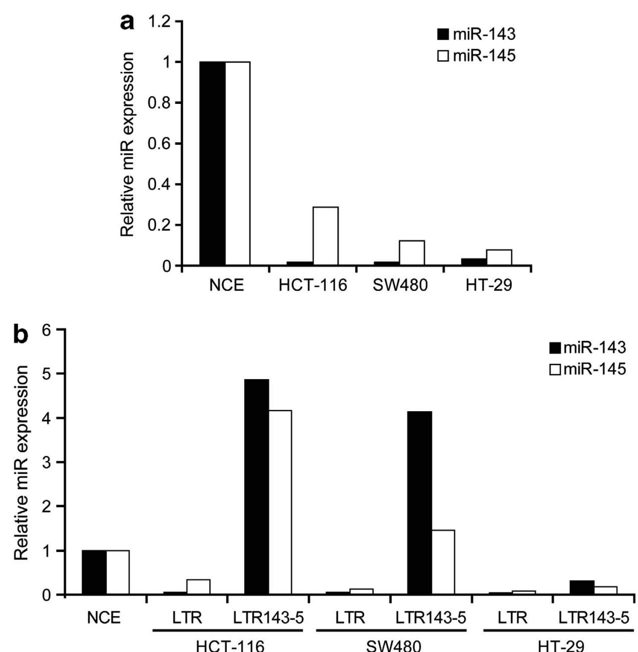

Figure 1. MiR-143 and miR-145 expression is downregulated in CRC cell lines. (**a**) Quantitative reverse transcriptase–PCR (qRT–PCR) analysis of the expression levels of miR-143 and miR-145 in CRC cell lines (HCT-116, SW480 and HT-29) and normal colonic epithelium (NCE) samples. (**b**) qRT–PCR evaluation of miR-143 and miR-145 expression in HCT-116, SW480 and HT-29 cell lines transduced with LTR or LTR143-5. Transduced cells were compared with NCE cells.

We then asked whether the restoration of miR-143 and miR-145 expression might act as a sensitizer to the treatment with 5-fluorouracil, irinotecan and oxaliplatin, the current therapeutic regimens for CRC. The results are shown in Figure 2d, where a dramatic decrease in cell viability after 72 h of treatment is observed. Indicating that miR-143 and miR-145 do not only affect proliferation of CRC cells, but also their survival, opening the possibility of them influencing a set of genes with pleiotropic properties.

MiR-143–145 cluster expression significantly decreases *in vivo* tumor growth

Having confirmed that the coordinated expression of the miR-143 and miR-145 transcripts provokes a decrease in the tumorigenic properties of HCT-116 *in vitro*, we endeavored to translate our findings in a tumor growth *in vivo* model. To this end, HCT-LTR143-5 and HCT-LTR were injected subcutaneously in immunocompromised mice, and the expression of the regulatable transgene was induced as soon as tumor formation was detectable. As shown in Figure 3 and Supplementary Table 1, the re-expression of miR-143 and miR-145 caused a significant deviation from the growth kinetic of control cells, suggesting that miR-143 and miR-145 have a major role in CRC progression.

Several miR-143 and miR-145 targets are involved in RAS signaling

To gain a further understanding of the mechanisms by which these miRNAs behave as oncosuppressors, we tried to establish whether any of their putative targets exerts a crucial function in colon cancer pathogenesis. We identified, among the miR-143 and miR-145 target genes predicted by Targetscan,[16] some transcripts

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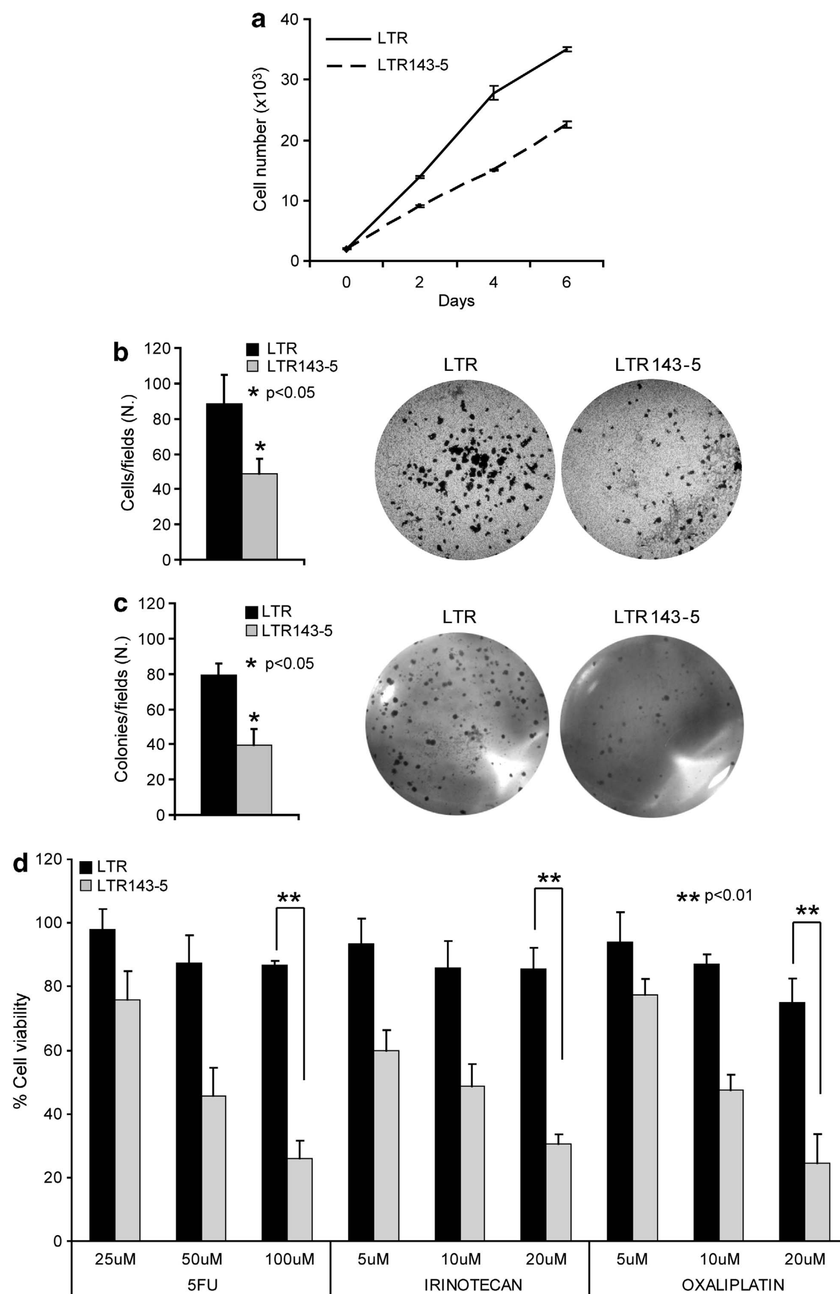

Figure 2. MiR-143 and miR-145 overexpression reduces cell growth, migration and colony formation ability, and influences the sensibility to chemotherapeutic drugs. (**a**) Growth curve of HCT-LTR or HCT-LTR143-5 treated with doxycyclin and flow sorted (day 0). (**b**) Number of migrated cells in HCT-116 transduced with LTR or LTR143-5, 48-h post-sorting. Right panel: representative contrast images. Scale bar $= 200\,\mu m$. (**c**) Colony formation of cells transduced as above, 16 days post-sorting. HCT-116 LTR and LTR143-5 cell lines in soft agar, after 16 days. Right panel: representative contrast images as above. (**d**) Chemosensitivity of HCT-116 cell lines overexpressing miR-143 and miR-145 compared with HCT-LTR cells after 72 h of treatment. In each panel, data are expressed as means $\pm$ s.d. of three independent experiments performed in triplicate.

that particularly hint at the role they have in CRC progression. Among these, KRAS, targeted by miR-143,[17] is activated by mutations in roughly 30% of CRC patients; BRAF (targeted by miR-143) is the immediate downstream effector of KRAS in the MAPK pathway, and is mutated in 10 % of CRC; KLF5 (targeted by miR-143 and miR-145) is a tissue-specific transcription factor, which mediates the transformation of normal epithelium by oncogenic KRAS;[13] erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ERBB3) (a target predicted for both miRNAs) stimulates the MAPK pathway through KRAS. Finally, CD44 (targeted by miR-143 and miR-145), has an important role in cell survival and migration, and is a direct target of p53 transcriptional repression.[14] Although the effect of miR-143 on KRAS in CRC tissues has already been described,[17] the remaining targets are novel. Notably, we found through Mirtar[18] that BRAF has a target site for miR-143 in the coding region, which is neglected by most target prediction engines (Supplementary Table 2) and yet, the presence of miRNA target sites in the coding region of genes was confirmed in previous reports.[19,20] We chose to assay the target protein levels 2 days after attaining full miRNA induction, in order to allow protein and transcript turnover to be affected by post-transcriptional repression. Results in Figure 4a show that KLF5, KRAS and BRAF

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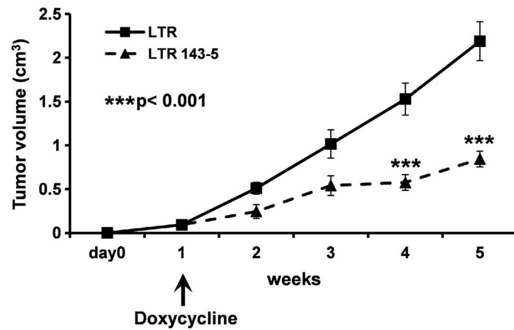

Figure 3. MiR-143 and miR-145 overexpression reduces the *in vivo* growth of tumors in NSG mice. Tumor growth curve in NSG mice subcutaneously injected in one flank with HCT-LTR and in the other with HCT-LTR143-5. Data are expressed as means ± s.e.m.

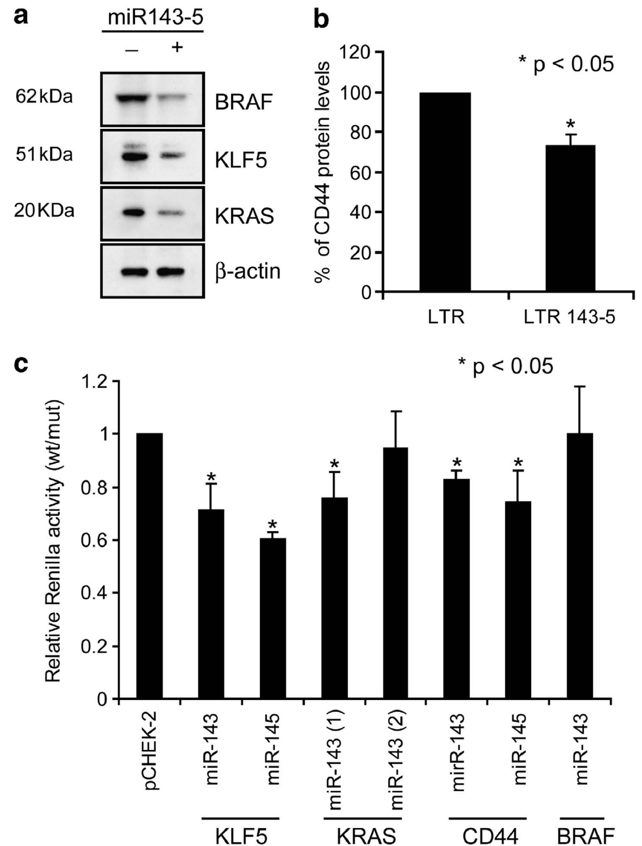

Figure 4. MiR-143 and miR-145 overexpression induce a decrease in the level of target protein. (**a**) Western blot analysis of KRAS, BRAF and KLF5 expression in HCT-LTR or HCT-LTR143-5, 5 days post-induction, β-actin is used as loading control. (**b**) Flow cytometry analysis of CD44 expression in HCT-LTR and HCT-LTR143-5, 5 days post-induction. Data are expressed as means ± s.d. of three independent experiments. (**c**) Reporter assay of wt and mutated target constructs. Histograms show normalized mean values of the relative renilla activity. Reporter activity is normalized to control plasmid. Data are expressed as means ± s.d. of three independent experiments.

target proteins are significantly diminished in cells where miR-143 and miR-145 are induced, albeit to a different extent that might arise from differential affinity of the miRNA for the transcript, differential transcript/protein half-life or other factors. As for the ERBB3 protein, we assayed its levels by flow cytometry, but it appeared to be unaffected by miR-143 and miR-145 induction (Supplementary Figure 2).

Another potential target of the oncosuppressive capabilities of miR-143 and miR-145 is CD44. It has been shown that CD44, a ligand for hyaluronic acid, mediates survival signals and is transcriptionally repressed by p53.[14] As miR-143 and miR-145 are upregulated by p53, and *in silico* predictions implicate these miRNAs in CD44 suppression, we examined the CD44 levels in cells overexpressing miR-143 and miR-145 by flow cytometry. Indeed, the expression of CD44 is diminished on overexpression of these miRNAs, confirming the targeting of CD44 by miR-143 and miR-145 (Figure 4b). In addition to the novel targets, we focused our attention on the previously established target genes that belong to the GFR–MAPK pathway, IRS-1 and extracellular signal-regulated kinase-5, confirming the downmodulation at the protein level for IRS-1 but not for extracellular signal-regulated kinase-5 (Supplementary Figure 3).

MiR-143 and miR-145 share targets in their regulatory network

We created luciferase-based reporter constructs bearing part of the target transcript 3′ untranslated region (UTR)/coding sequence (CDS) and co-transfected them in HCT-116 cells, along with vectors for miR-143 and miR-145 constitutive overexpression. We compared the activity of the target UTR/CDS construct with that of a cognate construct bearing a mutation in the 'seed' sequence[21] (Supplementary Table 3). We thus obtained the relative strength of inhibition for the miRNA/target site pair, showing that, in those instances where both miRNAs are predicted to target the same transcript, they downregulate the constructs with a similar efficiency (Figure 4c), providing the molecular basis for the assumption that miR-143 and miR-145, being co-regulated and sharing targets and affected pathways, can be considered as part of an integrated program of gene regulation. However, the site we identified in the BRAF transcript did not prove to be a genuine target site by this assay. The possibility exists that mechanisms other than direct post-transcriptional regulation operate to diminish the BRAF protein levels following miR-143 and miR-145 expression. Whether alternative assays to verify the site-specific interaction of the miRNA with the putative target sequence, such as TAP Tar[22] or the use of full-length, epitope-tagged transgenes, might lead to a positive response, has not been explored.

We also observed that a site elsewhere suggested as targeted by miR-143 (residues 3772–3779 of Genbank NM_033360) in LoVo cells[17] was not functional in our setting, (Supplementary Table 3), nor could we amplify by PCR on complementary DNA the region harboring it (data not shown). This suggests that the above transcript might be expressed as an alternative upstream polyadenylated form in HCT-116 cells that excludes the more distal mir-143 target site.

Phenotype rescue is not fully established through single target gene re-expression

The subset of target genes we identified is centered on the GFR–MAPK activation pathway, with the exception of CD44 being at least partly connected with the suppression of growth by p53. To ascertain whether a decrease in any of these proteins mediates the oncosuppressive properties of the miR-143–145 system, we performed the functional experiments described in Figure 2. but with the concomitant transduction of cells having lentiviral constructs that allow to restore single target genes, so as to observe whether partial or total phenotype recovery occurred (Supplementary Figure 4). The results shown in Figure 5a support the hypothesis that all target genes contribute to the oncosuppressive properties of miR-143 and miR-145, as their re-expression mitigates the decrease in proliferation imparted by these miRNAs. We also performed double coexpression experiments with KLF5

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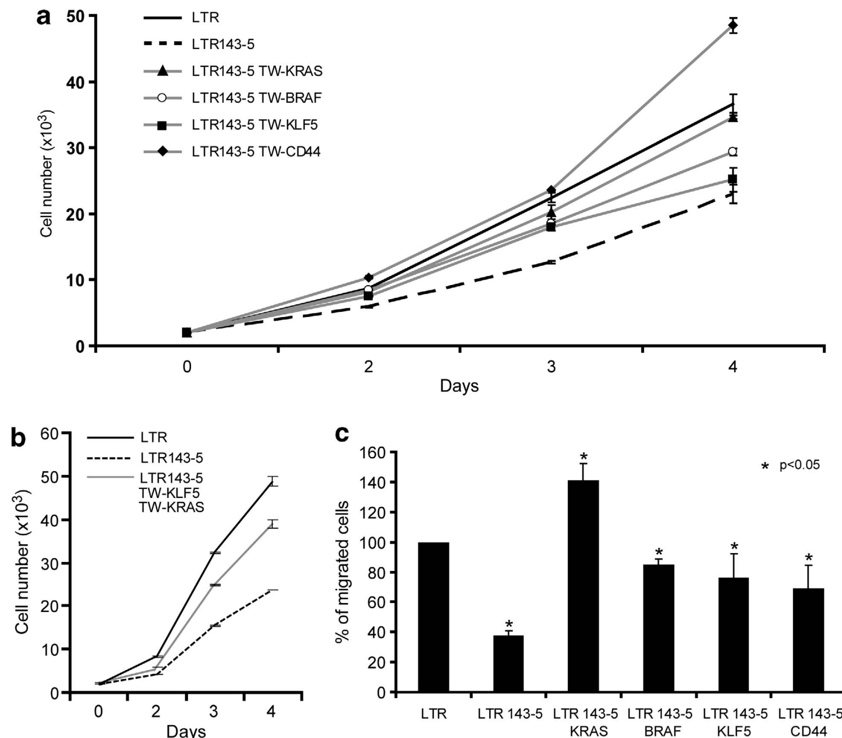

Figure 5. Target gene re-expression in HCT-LTR143-5 induces phenotypic rescue. (**a**) Growth curve of HCT-LTR and HCT-LTR143-5 expressing target genes as indicated treated with doxycycline and flow sorted (day 0). (**b**) Growth curve of HCT-LTR and HCT-LTR143-5 expressing both KRAS and KLF5. (**c**) Percentage of migrating cells in HCT-LTR and HCT-LTR143-5 as indicated in (**a**), 48-h post-sorting. In each panel, data are expressed as means ± s.d. of three independent experiments performed in triplicate.

and KRAS (Figure 5b), as a synergistic effect was expected based on previous evidence.[13] Even in this case, proliferation was not fully restored, suggesting that perturbation of the RAS–MAPK axis is not sufficient to explain the effect of miR-143–145 cluster on CRC cell proliferation (Figure 5b). When we examined the effect of target restoration on the migration capabilities of CRC cells overexpressing miR-143–145 cluster, we observed that restoring KRAS expression was sufficient to completely recover the ability of HCT-116 cells to migrate, whereas a weaker effect was seen with the overexpression of CD44, BRAF and KLF5 (Figure 5c). It might be inferred that the weak effect depends on non-physiological levels attained through the use of a strong promoter, and that a recovery in the proliferation capabilities might slightly affect the readout of the migration assay. However, as the duration of the assay is too short for the cells to show a sharp decrease in proliferation, and having assessed that the KRAS levels were not brought to a dramatic overexpression, we can rule this possibility out.

Global transcriptome changes associated with miR-143 and miR-145 expression

As only partial recovery was observed in the phenotype rescue experiments, we aimed at a more comprehensive description of the miR-143 and miR-145 regulatory network, in order to identify other mediators of their action on tumorigenic properties.

Recent evidence has been gathered supporting the notion that miRNAs act on their target gene repertoire at the transcript level, that is, not only by impairing the translation of the mRNA, but also by having a tangible effect on transcript stability.[23] This led us to characterize, at a global transcriptome level, the effects of miR-143 and miR-145 enforced expression in the context of CRC cell lines. To this end, we performed microarray analysis of RNA from HCT-LTR and HCT-LTR143-5. We hence examined the annotation for the most deregulated genes, both up- and downregulated (full microarray intensity values are available online in the 'Supplementary microarray data' file). A list of potential target mRNAs was obtained for both miR-143 and miR-145 using Targetscan 5.1.[16] Target genes were ranked using either total context score or probability of preferentially conserved targeting. The probability of a quantitative correlation between these scores and levels of transcript downmodulation were assessed using the Kendall tau correlation test. Kendall correlation of transcript modulation with total context scores was highly significant for both miR-143 and miR-145 predicted targets ($P = 0.1 \times 10^{-4}$ and $P = 1.32 \times 10^{-7}$, respectively), whereas no significant correlation was observed when using preferentially conserved targeting. These results suggest that, in this instance, the thermodynamics component of miRNA targeting may affect target modulation more than conservation of specific regions on the seed.

To detect significant variations in gene networks rather than in a single gene, we ran a Gene-Enrichment and Functional Annotation Analysis using the tools available in the DAVID website.[24,25] Using the DAVID tool, we searched among the transcripts that resulted deregulated, at least to a mild extent ($>$1.5-fold), in the miR-143–145 expressing cells for enrichment in specific pathways. We therefore analyzed 2422 unique identifiers corresponding to the down- or upregulated transcripts with the functional annotation clustering tool, with 607 genes assigned to pathways and three pathways enriched with a *P*-value below 0.01. In fact, we observed a sharp decrease in mediators of the G1/S transition of the cell cycle in agreement with the previously described decrease in proliferation rate (Supplementary Tables 4 and 5). Interestingly, all genes in these categories, with the exception of *CCND2*, displayed a decrease in mRNA levels, whereas genes upregulated in the miR-143 and miR-145 over-expressing cells did not belong to any statistically enriched pathway (data not shown). The pathways are linked with DNA

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replication and repair, as well as cell cycle progression, and most downregulated members of such pathways have a positive function. We speculate that the reduced activity of the GFR–RAS–MAPK pathway leads to the observed decrease in cell proliferation, which entails that a reduced fraction of cells express the genes that are required for DNA replication or for the G1 to S phase transition independently of a direct targeting by these miRNAs. Apart from the statistically significant modulated pathways, modulated genes were present in pathways related to p27 degradation, and in mediators of the GFR–phosphatidylinositol 3 kinase pathway (Supplementary Table 6).

DISCUSSION

Most reports on miR-143 and miR-145 did not consider them as interdependent genes, while our study examines the effect of their concomitant re-expression in cancer cell lines in which they are downregulated. Starting from the observation that they are frequently coexpressed, possibly indicating a coordinate action, we found that enhanced expression of miR-143 and miR-145 in CRC cells may lead to oncosuppression by altering the cells ability to proliferate, migrate, grow in an anchorage-independent manner and undergo apoptosis on genotoxic stimulation. These activities are suggestive that miR-143 and miR-145 belong to the family of oncosuppressive miRNAs and our experiment of *in vivo* xenotransplantation show indeed that their enhanced expression provokes a sensible delay in the growth of subcutaneous tumors. We further found that the reestablishment of miR-143 and miR-145 oncosuppressor functions in tumor cell lines can impose a proliferation block/delay that, on prolonged culturing, may eventually result in a counter-selection effect, in our case loss of transgene expression (data not shown). For this reason in this study, we used an inducible and controllable expression system. We propose that in studies of miRNAs with oncosuppressor functions, this approach may be more appropriate than a constitutive one for understanding the direct mechanism of their action.

In our study on the miR-143 and miR-145 regulatory action, we focused on targets chosen for being co-targeted by the two transcripts and for belonging to the GFR–MAPK signal transduction pathway. We found that, in addition to KRAS, a previously known miR-143 target, the two miRNAs downregulate KLF5, an important mediator, which synergizes with KRAS in colorectal transformation.[13] Their expression also accompanies a decrease in the BRAF protein, a component of the GFR–MAPK pathway downstream of KRAS. In addition, we found that both miRNAs target CD44, a receptor that transduces survival signals into the cell. To establish whether the decrease in one of these target proteins was sufficient to explain miR-143–145 cluster action, we expressed exogenous target genes along with the miRNAs. Given the intrinsic complexity of such an approach, we investigated the single contribution of the downregulation in target protein levels on the overall oncosuppression phenomena we observed. We have thus found that any decrease in single target proteins is involved to a different extent in the phenotype of growth suppression and, based also on published evidence, we conclude that miR-143 and miR-145 act in a concerted manner to suppress the GFR–MAPK signal transduction pathway at multiple levels.

It has been shown that the regulatory network of a miRNA comprises a small number of genes that are heavily affected at the protein level, and a larger group of genes that are mildly downregulated,[26,27] where secondary effects are to be counted as well. We have actually characterized to a much wider extent a possible spectrum of action of these miRNAs in the regulation of the transcriptome, observing an enrichment of genes belonging to cell cycle regulators among the downregulated transcripts. We also noted a statistically significant correlation of TargetScan-predicted target genes among the set of downmodulated transcripts genes, when such targets are ranked according to the total context score.

Although we believe that other miR-143 and miR-145 targets could be implicated in cell growth and survival, we propose that the concerted action of miR-143 and miR-145 on the epidermal growth factor-insulin-like growth factor (EGF-IGF) signaling pathway is wider than originally described and, coupled with the effect on the CD44 survival pathway, can account for a substantial part of the oncosuppression properties of this regulatory system. It is also becoming increasingly evident that the regulatory network of miR-143 and miR-145 comprises also feedback interactions of targets with the mechanism regulating these miRNAs expression: indeed both epidermal growth factor receptor[28] and KRAS[7,29] have been demonstrated to act as indirect determinants of miR-143 and miR-145 repression. Hence, our findings add to the already suggested notion that there is an intimate connection between these miRs and the epidermal growth factor receptor–MAPK signaling pathway.

As the re-expression of the miR-143 and miR-145 targets identified in this study, and belonging to the epidermal growth factor receptor–RAS–MAPK pathway is not sufficient to fully recover cell from the block in tumorigenicity, we sought to describe the full spectrum of molecular perturbations that occur after miR-143 and miR-145 expression. It is intriguing that many downregulated transcripts, among those we found enriched in predicted targets, belong to signaling pathways that mediate proliferation and survival signals.

MiRNAs have emerged as key players in post-transcriptional gene regulation, and as indispensable mediators of several important biological processes, including tumorigenesis and tissue homeostasis and regeneration.[30,31] We speculate that the full elucidation of their biological significance might benefit from an approach that focuses on targeted pathways, rather than on single genes. Such an approach is facilitated by the use of target gene predictions and expression databases, tools that are rapidly growing in their potential.

MATERIALS AND METHODS

Cell lines and cell cultures

CRC cell lines HCT-116, SW480 and HT-29 from ATCC were cultivated in the recommended media (see www.atcc.org for details).

The 293T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA). All media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen). Normal colonic mucosa samples were obtained from Sant'Andrea Hospital (Rome, Italy) on patients' informed consent and approval by the local ethical committee. Surgical specimens were washed several times with phosphate-buffered saline and subsequently, subjected to mechanical and enzymatic dissociation. The resulting cells were labeled with anti-epithelial antigen-fluorescein isothiocyanate (clone Ber-EP4, mouse immunoglobulin G_1, Dako, Milan, Italy) antibody and sorted with a FACS Aria (BD Biosciences PharMingen, San Jose, CA, USA).

Plasmid constructs and lentivirus infection

prTTA-Neo was obtained by cloning the reverse tet transactivator and a neomycin-resistance cassette in tandem into the lentiviral backbone of pRRLCMV. pTR-puro was obtained by cloning the tet repressor in tandem with a puromycin-resistance cassette into the lentiviral backbone of pLentilox 3.7.[32] pLTR (Lentiviral-Tet-inducible-RFP) was obtained by inserting, in a the pLentilox 3.7 lentiviral backbone, the inducible promoter from pUHD10.3[33] driving the transcription of RFP. In pLTR143-5, the miR-143–145 precursor DNA is cloned in the 3′ UTR of RFP. The following primers were used: forward 5′-TGCCATTGTTTGCACAACTT-3′; reverse 5′-CAAGAGTACGGCAGTGCTGA-3′ positioned at −298 and +198 relative to cluster limit, respectively. Tetracycline-responsive cells were obtained by transduction with prTTA-Neo and a pTR-Puro. Following antibiotic selection, cells were transduced with pLTR or its derivative

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pLTR143-5, induced with the tetracycline analog doxycycline and, after 2 days, RFP-positive cells were flow sorted.

In the rescue experiments, HCT-116 were infected with lentivirus constructs obtained by cloning target gene CDS in pTWEEN.[34] In particular BRAF, CD44 and KLF5 sequence were cloned in pTween–GFP vector, whereas KRAS was cloned in pTween–CFP vector. CD44 and BRAF complementary DNAs were obtained as vectors pBabePuro-CD44 and pBabePuro-BRaf (Addgene, Cambridge, MA, USA) and subcloned in pTween. KRAS and KLF5 sequence were PCR amplified from human genomic DNA. The following primers were used: KRAS forward 5′-ggcctgctgaaaatgactgaat-3′; KRAS reverse 5′-attataatgtaatctgggtgtt-3′; KLF5 forward 5′-cccctcgagtgcccatggctacaa-3′; KLF5 reverse 5′-gggcagcgct cagttctggtgcc-3′. For gene reporter assays, oligonucleotides harboring the target site were subcloned into the dual reporter plasmid psiChek-2 (Promega, Madison, WI, USA). All the constructs were verified by Sanger sequencing.

Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Fifty nanograms of RNA were reverse transcribed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Paisley, UK).

Quantification was performed with TaqMan miRNA assays (Applied Biosystems) for miR-143 and miR-145 and RNU6B was chosen as reference.

Cell growth, migration and colony formation assay

HCT-LTR and HCT-LTR143-5 cells were plated at a density of 2×10^4/ml in 96-well plates in triplicate. Cell proliferation was monitored by counting the cells and confirmed by using the CellTiter-Blue Viability Assay (Promega).

The motility of transduced HCT-116 was evaluated in 24-well transwell chambers (Corning Life Sciences, Corning, NY, USA), as previously described.[35]

Assays of colony formation in soft agar were done as previously described.[35]

Cytotoxicity assay

HCT-LTR and HCT-LTR143-5 were plated at a density of 2×10^4/ ml in a 96-well plate in triplicate. Twenty-four hours after seeding, cells were treated with different concentrations of the indicated chemotherapeuticals. After 72 h of treatment, cell viability was assessed using CellTiter-Glo assay (Promega) following the manufacturer's instructions.

Xenograft mouse models

In all, 1×10^5 cells, resuspended in matrigel, were subcutaneously injected in the flanks of 20 SCID/IL-2R null (NSG) mice. HCT-LTR cells were injected in one flank, whereas HCT-LTR143-5 cells were injected in the other. Doxycycline administration in drinking water (200 μg/ml) started after tumor detection. Tumor size was monitored by measuring the length (L) and width (W) with a caliper. Volumes were calculated by using the following formula: $(L \times W^2) \times 0.52$ and statistic significance was calculated using one-way analysis of variance by GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). All animal experiments were conducted after approval of the competent authorities.

MiRNA target prediction

TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org) and MirTar (http://mirtar.mbc.nctu.edu.tw/human/) were used for miR-143 and miR-145 target prediction.

Western blot analysis

Protein expression was analyzed by standard immunoblot procedure using anti-KRAS, anti-BRAF and anti-BTEB2 (KLF5). All primary antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-β-actin monoclonal antibody was used as a loading control (Oncogene Research Products, La Jolla, CA, USA).

The quantization of protein expression was determined after normalization to β-actin, by measuring the optical density of respective band blots using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry

Transduced HCT-116 cells were incubated with the specific antibody fluorescein isothiocyanate-conjugated anti-CD44 (R&D Systems, Minneapolis, MN, USA), phycoerythrin-conjugated anti-ERBB3 (R&D Systems) or isotype-matched control antibodies. Fluorescence intensity was evaluated by a FACSCanto flow cytometer (BD Biosciences).

Reporter assay

HCT-116 were co-transfected with 0.2 μg of reporter vectors and 1.8 μg of miR-143–145 expression vector. Luciferase activity was detected 48 h after transfection using the dual luciferase assay (Promega). Histograms show normalized mean values of the relative renilla activity, calculated by dividing the renilla luciferase values (normalized by firefly luciferase values) obtained in 3′ UTR/CDS binding site wt vector (psiCheck-2) by those mutated, from three independent transfections.

Gene array

Gene array was performed as previously described.[35] Briefly, total RNA was extracted from HCT-LTR and HCT-LTR143-5 transduced cells. RNA was labeled and hybridized to the Affymetrix GeneChip1.0ST array (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Hybridization values were normalized by the Robust Multi-array Average (RMA) method. Genes were filtered for a logarithmic expression value of at least 8 and the ratio between HCT-LTR and HCT-LTR143-5 was selected to examine genes with a differential expression of at least 1.5-fold.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grant from Associazione Italiana per la Ricerca sul Cancro, AIRC (Start-up 6326 to LR-V and Regional Fellowship to CV). We thank Tania Merlino for contribution in editing the manuscript.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

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