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Original Articles

Sequence diverse miRNAs converge to induce mesenchymal-to-epithelial transition in ovarian cancer cells through direct and indirect regulatory controls

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ABSTRACT

Epithelial-to-mesenchymal transition (EMT) has been shown to be similarly regulated by multiple miRNAs, some displaying little or no sequence identity. While alternate models have been proposed to explain the functional convergence of sequence divergent miRNAs, little experimental evidence exists to elucidate the underlying mechanisms involved. Representative members of the miR-200 family of miRNAs and the sequence divergent miR-205 miRNA were independently over expressed in mesenchymal-like ovarian cancer (OC) cells resulting in mesenchymal-to-epithelial transition (MET). The miR-205 and the miR-200 family of miRNAs were found to coordinately induce MET in mesenchymal-like OC cells by affecting both direct and indirect changes in the expression of genes previously associated with EMT/MET. Only two direct targets of these miRNAs (*ZEB* 1 and *WNT5A*) are commonly down regulated in response to over-expression of miR-205 and/or the miR-200 family of miRNAs. Down-regulation of these genes, alone or in combination, only partially recapitulates the changes induced by the miRNAs indicating an additional contribution of indirect changes regulated by the miRNAs. Combined gene expression analyses and phylogenetic comparisons suggest an evolutionarily more recent involvement of miR-205 in the EMT/MET process.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) and mesenchymal-to-epithelial transition (MET) are reciprocal molecular processes essential in early embryonic development that have been co-opted by cancer cells to facilitate tumor metastasis [1]. Recent studies have demonstrated that both EMT and MET can be induced by modulations in cellular levels of microRNAs (miRNAs), a well-studied class of small regulatory RNAs [2].

The miR-200 family of miRNAs (miR-200a, −200b, −200c, −141, −429) has been shown to play crucial roles in cancer initiation and metastasis and to have significant potential as cancer therapeutic agents [3]. Our laboratory has been particularly interested in the ability of members of the miR-200 family of miRNAs to induce MET when ectopically over expressed in mesenchymal-like ovarian cancer (OC) cells. For example, we have previously shown that ectopic over expression of miR-200 family members in a variety of mesenchymal-like OC cells (HEY, HEYA8, SKOV3) is capable of inducing both morphological and molecular changes (mRNA and protein levels) characteristic of MET [3].

Sequences located within miRNA “seed regions” (typically situated at positions 2–7 from the miRNA 5′-end) are the predominant determinant of miRNA regulatory control [4]. Given that even a single nucleotide substitution within the highly conserved seed region of miRNAs can dramatically change the spectrum of regulated mRNA target genes [5], it is remarkable that families of miRNAs sequence divergent from one another can similarly regulate the EMT/MET process in a diversity of cancer cells. For example, miR-205, although highly sequence divergent from members of the miR-200 family in both seed and non-seed regions (Fig. 1B), has been reported to display a similar ability to induce MET in mesenchymal-like lung [6] and prostate [7] cancer cells. The goal of the present study is to employ a

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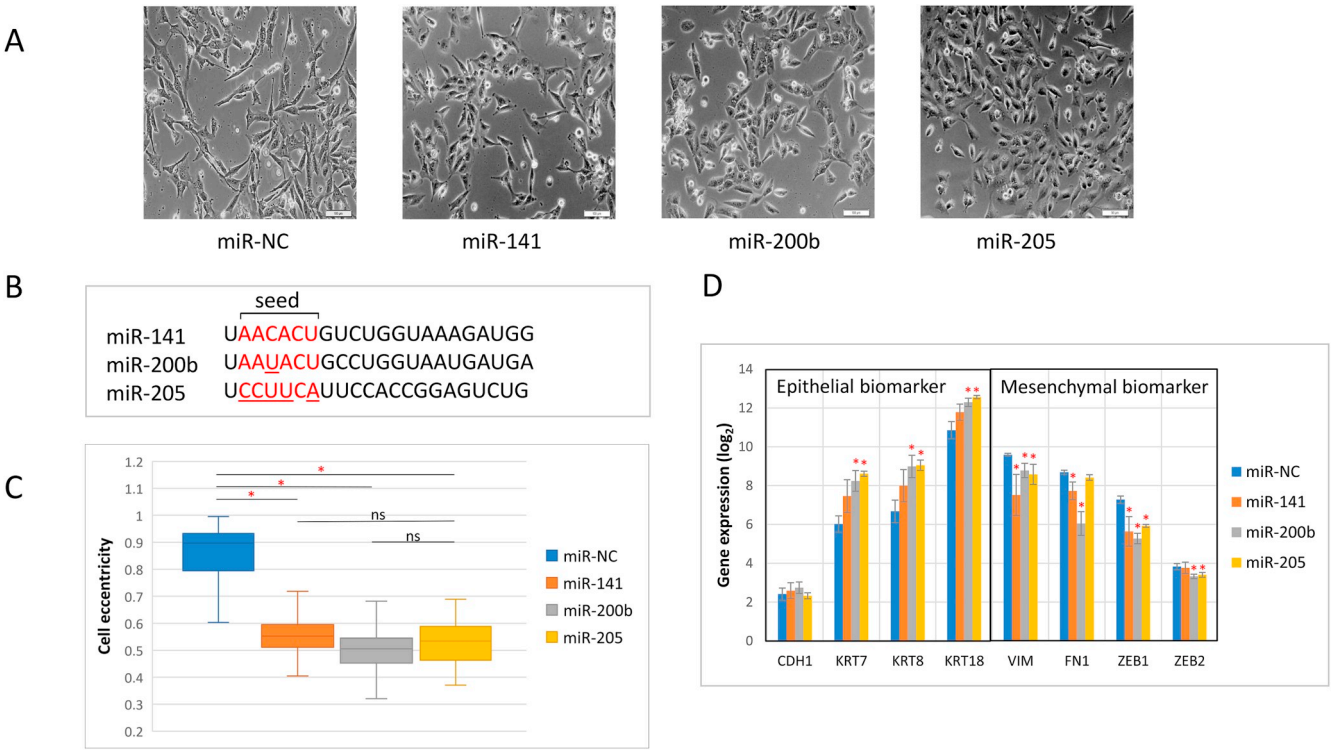

Fig. 1. miR-205 over expression induces morphological changes characteristic of MET indistinguishable from those induced by the sequence divergent miR-200 family of miRNAs. (A) Representative microscopic images of HEY cells 48 h post transfection with miR-141, miR-200b and miR-205 and miR-NC (negative control) (While scale bars = 100 μm). (B) Sequence alignments of miR-141, miR-200b, and miR-205. Seed regions are colored in red. Differences in seed region of miRNAs relative to miR-200b are underlined. (C) Ectopic over expression of miR-141, miR-200b and miR-205 induced nearly identical morphological changes characteristic of MET (elongated to cuboidal) 48 h post-transfection. 300 cells are randomly selected from each of three biological replicates to compute the eccentricity value of each miRNA transfection group. (Error bars represent the minimum and maximum of the eccentricity values in each group (cell eccentricity: 0 = circular shape, 1 = linear shape; * = $p < 0.05$, student t-test). (D) Changes in expression (relative to negative controls) of representative epithelial and mesenchymal biomarkers post 48 h after ectopic over expression of miR-141, miR-200b and miR-205 (Error bars represent the standard deviation of the gene expression values between the three biological replicates; * = p-value < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

combination of molecular and computational techniques to explore how miRNAs with little to no sequence homology acquire the ability to regulate identical cellular functions relevant to cancer onset and progression. A full understanding of the complexities of miRNA regulatory control is prerequisite to the successful future application of miRNAs as cancer therapeutic agents.

We report here that although miR-205 has little to no sequence homology with members of the miR-200 family of miRNAs, it induces morphological transitions in mesenchymal OC cells that are morphologically indistinguishable from those induced by members of the sequence divergent miR-200 family. We show that miR-205 and the miR-200 family of miRNAs coordinately induce MET in mesenchymal-like OC cells by affecting both direct and indirect changes in the expression of genes previously associated with EMT/MET. While two direct targets of these miRNAs (*ZEB* 1 and *WNT5A*) are commonly down regulated in response to over expression of miR-205 and/or the miR-200 family of miRNAs, down regulation of these genes alone or in combination only partially recapitulate the changes induced by the miRNAs further underscoring the importance of indirect regulatory effects. Finally, evidence is presented indicating that regulation of EMT/MET associated genes in humans by miR-205 is likely the result of convergent evolution initiated by the clustering of miR-205 with members of the miR-200 family on chromosome 1–80 MYA [8].

2. Materials and methods

2.1. Cell culture and transfection

The HEY cell line [9] was kindly provided by Gordon Mills, Knight Cancer Institute, Oregon Health & Science University. Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (Fetal Bovine Serum; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic solution (Mediatech-Cellgro). 1×10^5 cells were seeded per well in 6-well plates. For miRNA transfection, cells at exponential phase of growth were transfected with 33 nM Pre-miR miRNA Precursors (Life Technologies, Carlsbad, CA) using Lipofectamine 2000 (Life Technologies). Pre-miR miRNA Precursor Negative was used as negative control. For siRNA transfection, cells at exponential phase of growth were transfected with 33 nM Silencer Select (Life Technologies) using Lipofectamine 2000 (Life Technologies). Silencer Select Negative Control siRNA was used as a negative control. Cells were allowed to grow for 48 h before RNA extraction.

2.2. Microarray analysis

Cells (3 biological replicates) were harvested at 48 h after transfection and RNA extracted and subjected to microarray analysis (Affymetrix, U133 Plus 2; Santa Clara, CA) as previously described [8]. Robust Microarray Averaging (RMA) was used for normalization of microarray signal and summarization using the Affymetrix Expression Console Software (Version 1.4). Expression data analysis was carried

out using the limma statistical package executed using the R software suite [10]. Differentially expressed mRNAs were identified through fold change and FDR adjusted p-value calculated using moderated *t*-test.

2.3. Gene set similarity measurement

The cosine similarity index was used to determine the similarity between sets of genes (*e.g.*, genes differentially expressed after over expression of different miRNAs) [11].

2.4. miRNA target prediction

Prediction algorithms were downloaded and implemented (miRanda [12] - (www.microrna.org), miRDB [13] - (http://mirdb.org/download.html), and TargetScan [14] - (http://www.targetscan.org/vert_72/) according to the recommended procedures.

2.5. Image analysis

Morphological changes were monitored using an Olympus IX51 microscope (Olympus Optical, Melville, NY) and quantified using CellProfiler cell-imaging software (2.1.0) [15].

2.6. Real-time PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (RNeasy, Qiagen, Germantown, MD). Four micrograms of RNA were reversed transcribed into cDNA using the Superscript III First-Strand Synthesis System (Life Technologies) according to the manufacturer's instructions. Real-time PCR was performed using TaqMan Real-Time PCR Master Mixes (Life Technologies) on a CFX96 Real-Time System (Bio-Rad, Hercules, CA). Expression values were normalized using *GAPDH* as a reference gene. Normalization and fold-change were calculated using the ΔΔCt method.

2.7. Western blot

The total protein concentration of the supernatant was determined using a protein assay reagent kit (Bio-Rad). To the lysates, equal volumes of 2X Laemmli sample buffer were added and the samples were heated to 90 °C for 5 min. Equal amounts of proteins were separated by 4–20% gradient precast TGX gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in 10 mM Tris-buffered saline. After blocking, the membranes were probed with the primary antibody overnight at 4 °C with gentle shaking. Antibodies used are against Zinc Finger E-Box Binding Homeobox 1 (ZEB1 antibody, Cat # 3396, Cell Signaling Technology, Danvers, MA), Wnt Family Member 5A (WNT5A antibody, Cat # 2392, Cell Signaling Technology), and Beta-actin (ACTB antibody Cat # 5441, Sigma, St. Louis, MO). After incubation with the corresponding anti-mouse- or anti-rabbit-horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX), the Western blots for specific proteins were detected using an enhanced chemiluminescence (ECL) kit (Life Technologies) and images were developed by Amersham Imager 600 (GE Healthcare, Chicago, IL) according to the manufacturer's instructions.

3. Results

3.1. miR-205 over expression induces morphological changes characteristic of MET indistinguishable from those induced by the sequence divergent miR-200 family of miRNAs

We have previously reported that ectopic over expression of miR-200 family members in mesenchymal-like OC cells results in the induction of morphological and molecular changes characteristic of MET [16–18]. We were interested in determining if ectopic over expression of miR-205 could induce similar changes despite being highly sequence divergent from miR-200 family members (Fig. 1B). Towards this end, we conducted a series of experiments where two representative members of the miR-200 family (miR-141 and miR-200b) and miR-205 were individually ectopically over expressed in HEY cells. The mesenchymal-like HEY cell line was originally derived from a human OC xenograft (HX-62) originally grown from a peritoneal deposit of a patient with moderately differentiated papillary cystadenocarcinoma of the ovary [9]. HEY cells were selected for use in this study because they have been previously shown to display morphological and molecular (mRNA and protein) responses to miR-200 family over expression characteristic of MET [3].

The results presented in Fig. 1A demonstrate that over expression of miR-205 in HEY cells induces changes in OC HEY cells characteristic of MET that are visually indistinguishable from those induced by the two representative members of the miR-200 family. Cell eccentricity analyses [15] of 300 randomly selected cells confirm that there is no statistically significant difference between the morphological changes induced by over expression of miR-205 and the two representative members of miR-200 family (Fig. 1C). Coordinated increases in the expression of representative epithelial biomarkers (KRT7, KRT8, KRT18) and decreases in the expression of mesenchymal biomarkers (VIM, FN1, ZEB1, ZEB2) are consistent with mesenchymal-to-epithelial transitions in response to over expression of each of the miRNAs (Fig. 1D).

3.2. The majority of changes in gene expression commonly induced by ectopic over expression of the miR-200 family miRNAs and miR-205 are the result of indirect regulatory controls

In an effort to better understand the molecular changes mediated by over expression of miR-200 family (miR-141 and miR-200b) and miR-205 miRNAs relative to controls, we conducted microarray gene expression analyses (Affymetrix, U133 Plus 2.0 Array; Additional file 1) as previously described [9]. We focused initially on changes in gene (mRNA) expression induced in common by ectopic over expression of the two representative members of the miR-200 family. Of the 6416 genes significantly differentially expressed in response to ectopic over expression of the miR-200 family members, 1994 genes were induced in common (Fig. 2A). The cosine similarity index of differentially expressed genes between the miR-200 family members is 0.51. This value of similarity was increased when comparing differentially expressed genes between miR-205 and miR-141/miR-200b. 3453 genes were induced in common between miR-205 and miR-141, with a similarity index of 0.65 (Fig. 2B), while 2197 genes were induced in common between miR-205 and miR-200b, with a similarity index of 0.59 (Fig. 2C). Overall, 91.3% (1821/1994) of the genes differentially expressed by ectopic over expression of both miR-200 family miRNAs were also significantly differentially expressed in response to over expression of miR-205 (Fig. 2D).

The fact that the miR-200 family and miR-205 miRNAs are highly sequence divergent makes it unlikely that they directly target a high proportion of the same genes. We confirmed this by employing three independent prediction algorithms (miRanda [12], miRDB [13], TargetScan [14]) to identify direct target genes of the three miRNAs analyzed in this study (Additional files 2–4). For example, according to the miRanda miRNA target prediction algorithm [12], 3943, 4027, and 2941 genes are predicted to be direct target genes of miR-141, miR-200b, and miR-205, respectively. Among these, 1440 genes are predicted to be direct target genes of both miR-141 and miR-200b, but only 444 out of these 1440 genes (30.8%) are predicted to be direct target genes of miR-205 (Supplementary Fig. S1).

Overall, our gene expression analysis, demonstrates that of the 1994 genes displaying a significant change in expression in response to over expression of the miR-200 family miRNAs, only 181 genes (9.1%) are

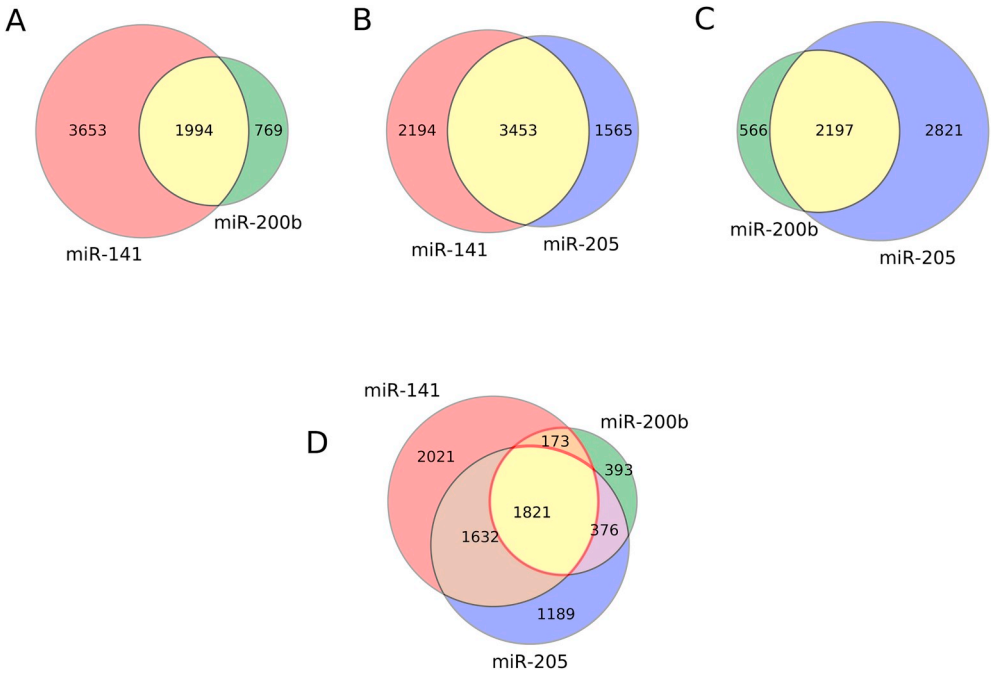

Fig. 2. Analysis of differentially expressed genes after transfection of miR-200 family members (miR-141 and miR-200b) and miR-205. (A) Venn diagram showing the intersection of differentially expressed genes between miR-141 and miR-200b. (B) Venn diagram showing the intersection of differentially expressed genes between miR-141 and miR-205. (C) Venn diagram showing the intersection of differentially expressed genes between miR-200b and miR-205. (D) Venn diagram showing the intersection of differentially expressed genes between miR-141, miR-200b, and miR-205. Differentially expressed genes between categories were filtered (FDR < 0.05 and fold change > 1.5).

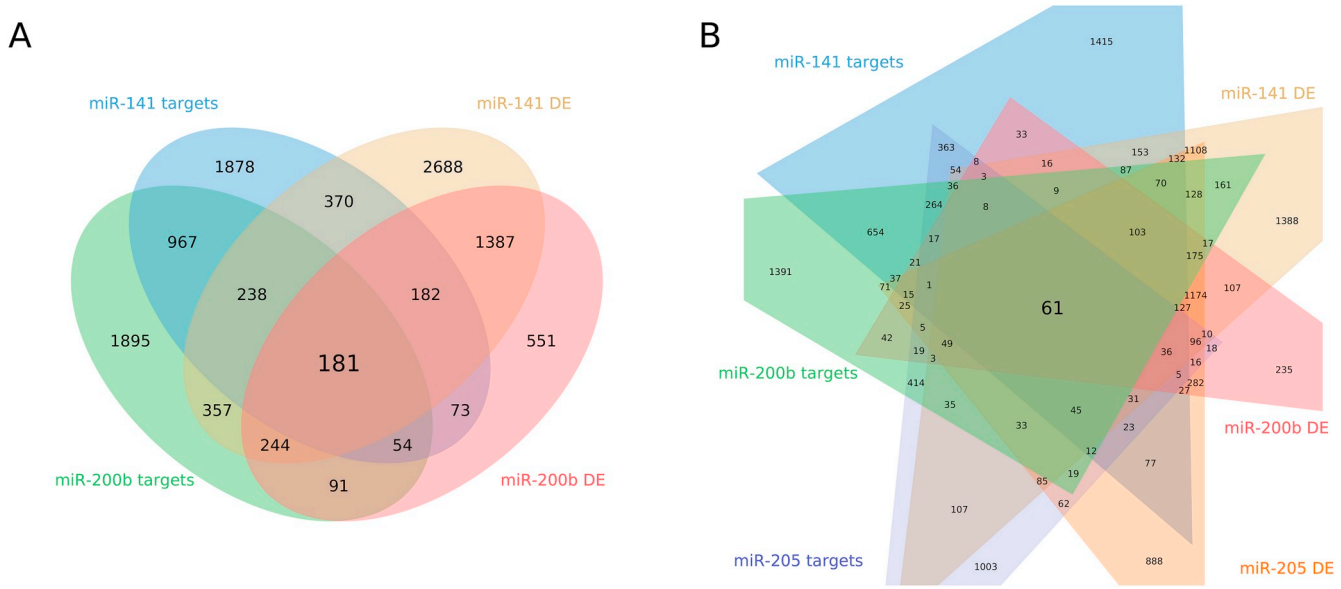

Fig. 3. Analysis of the intersection between differentially expressed genes and miRNA target genes after transfection of miR-200 family members (miR-141 and miR-200b) and miR-205. (A) Venn diagram showing the intersection of differentially expressed genes and miRanda-predicted miRNA target genes between miR-141 and miR-200b. (B) Venn diagram showing the intersection of differentially expressed genes and miRanda-predicted miRNA target genes between miR-200 family members (miR-141 and miR-200b) and miR-205. Differentially expressed genes between categories were filtered (FDR < 0.05 and fold change > 1.5).

predicted to be direct target genes of both of these miRNAs (Fig. 3A). Of the 1821 genes induced in common by the miR-200 family miRNAs and miR-205, only 61 (3.3%) are predicted to be direct target genes of all of these miRNAs (Fig. 3B). These results indicate that the vast majority of the changes in gene expression commonly induced by ectopic over expression of the miR-200 family miRNAs and miR-205 are the result of indirect regulatory effects.

3.3. The majority of changes in expression of EMT/MET-associated genes commonly induced by ectopic over expression of the miR-200 family miRNAs and miR-205 are also the result of indirect regulatory controls

We were next interested in determining if similar trends apply to a subset of genes previously identified as being directly involved in EMT/MET. For these studies, we focused our analysis on 84 genes that have been previously associated with the EMT/MET process [20]. Thirty-eight of these 84 genes were significantly differentially expressed by ectopic over expression of at least one of the miR-200 family members (miR-141 and miR-200b) or miR-205 (Fig. 4). Of these 38 genes, 17 (44.7%) were commonly induced by both members of the miR-200 family tested. Interestingly, 15 out of these 17 genes (88.2%) are also differentially expressed when miR-205 is included in the analysis. Of the 17 EMT/MET genes induced in common by the miR-200 family members, only four (23.5%) are predicted to be direct target genes of these miRNAs. Likewise, of the 15 EMT genes induced in common by the miR-200 family and miR-205 miRNAs, only two (13.3%) genes

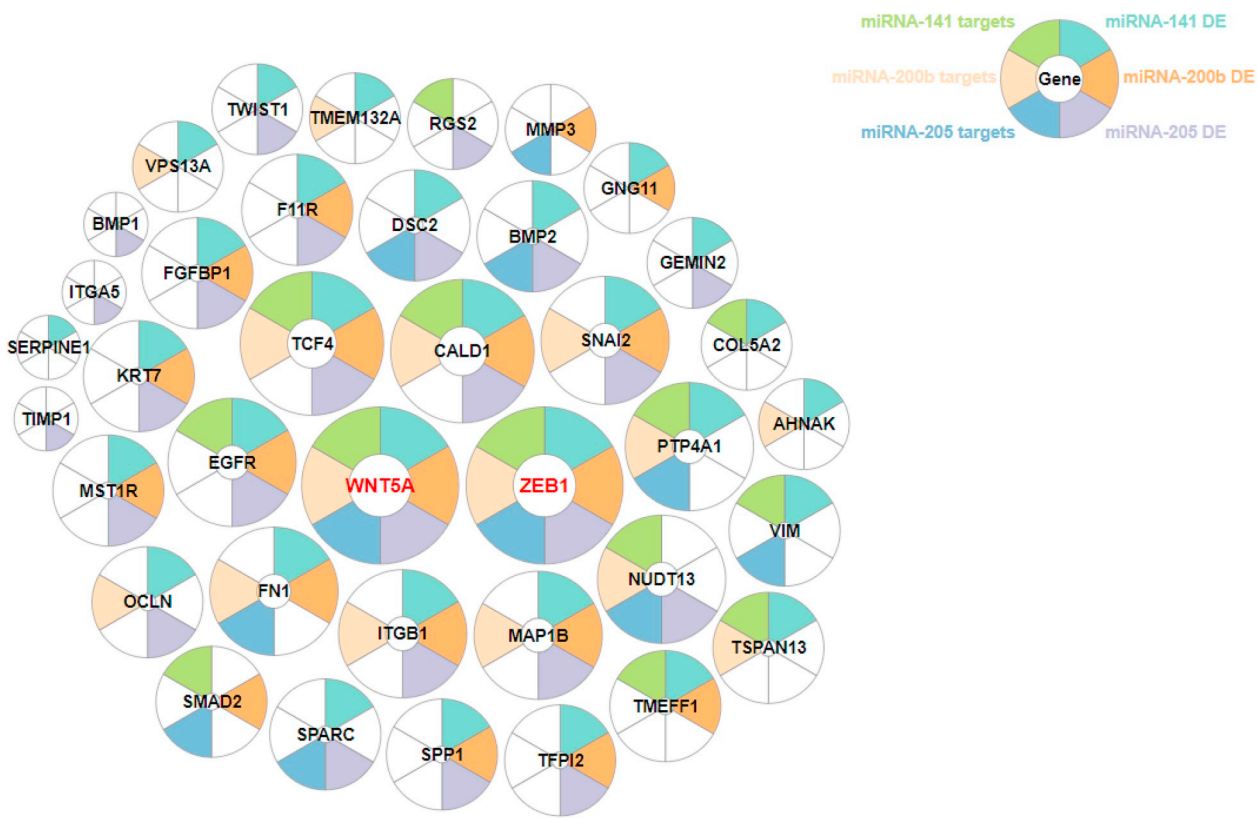

Fig. 4. Analysis of the intersection between differentially expressed EMT/MET genes and miRNA target genes after transfection of miR-200 family members (miR-141 and miR-200b) and miR-205. Thirty-eight EMT/MET genes were significantly differentially expressed by ectopic over expression of at least one of the miR-200 family members (miR-141 and miR-200b) or miR-205. Each circle represents a gene. The right half of the circle represents if the gene has been differentially expressed (DE) after transfection of miR-141 (right-top), miR-200b (right-middle), or miR-205 (right-bottom) in HEY cells after 48 h transfection. The left half of the circle represents if the gene is a direct target gene of miR-141 (left-top), miR-200b (left-middle), or miR-205 (left-bottom). The white slice of the circle means either this gene is not differentially expressed after transfection, or this gene is not the direct target of the corresponding miRNA. The non-white slice of the circle means either this gene is differentially expressed after transfection, or this gene is the direct target of the corresponding miRNA. Genes highlighted in red are the EMT/MET genes that are differentially expressed direct target genes of both miR-200 family members (miR-141 and miR-200b) and miR-205. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(*ZEB1 and WNT5A*) are predicted to be directly targeted in common by these miRNAs (Fig. 4).

3.4. Knockdown of ZEB1 and/or WNT5A induces intermediate morphological changes in HEY cells

Of the EMT-associated genes directly targeted in common by miR-141, miR-200b and miR-205, only *ZEB1* and *WNT5A* were consistently down regulated after ectopic over expression of these miRNAs (Supplementary Fig. S2A). This raises the possibility that reduced expression of these genes may alone be responsible for the morphological changes associated with over expression of the miRNAs. To test this hypothesis, we ectopically over expressed siRNAs against ZEB1 and WNT5A (alone and in combination) in HEY cells. While knockdown of ZEB1 and WNT5A, alone or in combination, resulted in a significant decrease in the expression of these genes on both the mRNA (Fig. S2B) and protein levels (Fig. S2C), the cell eccentricity analyses [15] indicated a non-significant overall difference in cell morphology from the negative controls (Fig. 5 A, B). However, detailed examination of cell images in the ZEB1/WNT5A knockdown groups reveals that some cells clearly exhibit a cuboidal, epithelial-like phenotype after treatment indicative of a partial or intermediate induction of MET (*e.g.*, note cells circled in yellow in Fig. 5A).

3.5. Knockdown of ZEB1 and/or WNT5A induces gene expression changes in HEY cells indicative of partial MET

To determine if the apparent intermediate level of morphological changes associated with knockdown of ZEB1/WNT5A may be similarly reflected on the molecular level, we employed a previously established analytical method that assigns an EMT score based on relative gene expression levels of a 16-gene panel of canonical EMT genetic markers [21]. In order to get the expression levels of these 16 genes, we conducted microarray gene expression analyses of three biological replicates of each treatment (Affymetrix, U133 Plus 2.0 Array; Additional file 5) as previously described [19]. EMT scores are calculated by summing the mesenchymal associated gene expression values minus the epithelial associated gene expression values for each replicate (*i.e., VIM + CDH2 + FOXC2 + SNAI1 + SNAI2 + TWIST1 + FN1 + ITGB6 + MMP2 + MMP3 + MMP9 + SOX10 + GCS − CDH1 − DSP − OCLN*). Cells with higher ranked EMT scores are considered more mesenchymal-like while those with lower scores are considered more epithelial-like [21] (Fig. 6 A). Consistent with the results of our morphological analyses, the ZEB1/WNT5A knockdown groups were found to consistently display intermediate EMT scores between the miR-200b, miR-141, and miR-205 over-expression groups (Fig. 6 B) and the negative control. While the EMT score associated with ZEB 1 knockdown (4.19) is significantly different from the negative control (6.02, $p < 0.05$), the EMT scores associated with si-ZEB1-WNT5A (4.56) or si-WNT5A (4.59) knockdown, although consistently lower, are not significantly different from the negative control. Of the three miRNAs over-expressed in HEY

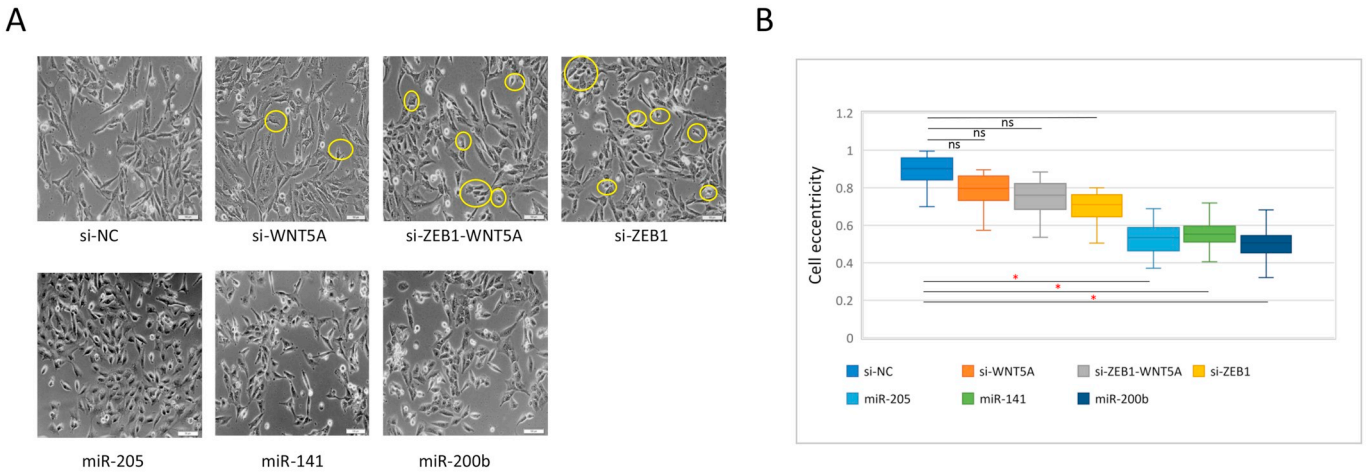

Fig. 5. Overexpression of miR-200 family (miR-141/miR-200b) or miR-205 miRNAs result in changes in HEY cell morphology characteristic of MET. (A) Representative microscopy images after 48 h transfection with ZEB1 and/or WNT5A siRNAs or miR-141, 200b, or 205. The representative epithelial-like cells in the ZEB1 and/or WNT5A knockdown groups are circled in yellow. (B) Boxplots showing the median of cell eccentricity of ZEB1 and/or WNT5A knockdown groups is consistently lower than the cell eccentricity of the negative control group, but higher than the median of cell eccentricity of the miR-200 family or miR-205 over expression groups. [ns: non-statistically significant from the negative control group; asterisks: statistically significant differences from the negative control group. (Error bars represent the minimum and maximum of the eccentricity values in each group; *p-value < 0.05)]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

cells, miR-200b is associated with the lowest average EMT score (2.46) followed by miR-141 (3.21) and miR-205 (3.97) all of which are significantly lower than the negative control (Fig. 6 B).

3.6. miR-205 targets fewer EMT/MET associated genes than members of the miR-200 family suggesting an evolutionarily more recent role in the EMT/MET process

The fact that miR-205 over expression is associated with the highest EMT score of the miRNAs tested is correlated with the fact that miR-205 is predicted to target relatively fewer EMT/MET associated genes than the miR-200 family (miR-200b, miR-141) (Additional file 6). Consider, for example, the representative EMT/MET pathway depicted in Fig. 7. While miR-200b and miR-141 are predicted to directly target 30 and 28 EMT/MET pathway genes, respectively, miR-205 is predicted to target only 17 genes. Collectively, these and similar results (Additional file 7) suggest that miR-205's involvement in the EMT/MET process may be evolutionarily more recent than the involvement of the miR-200 family.

Consistent with this hypothesis, miR-200b has been shown to be involved in EMT/MET associated processes in the mouse [22,23] while the limited studies on miR-205's role in mouse development is reported to be more narrow in scope [24]. Moreover, while miR-200b and miR-205 map to the same chromosome in humans (human chromosome 1) and are under similar regulatory control [25], miR-205 maps to a distinct chromosomal location (mouse chromosome 1) from miR-200b (mouse chromosome 4) in mice. Collectively, these observations suggest an evolutionarily more recent involvement of miR-205 in the EMT/MET process possibly facilitated by a chromosomal translocation event

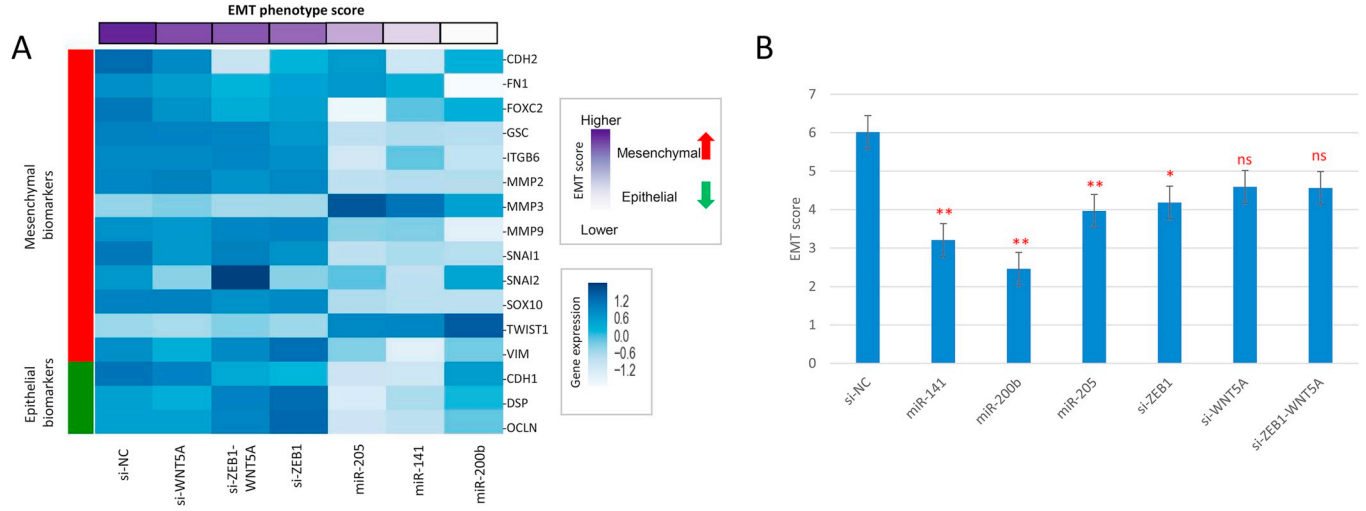

Fig. 6. Changes in EMT scores induced by miR-200 family (miR-141/miR-200b) or miR-205 miRNAs are partially recapitulated by ZEB1 and/or WNT5A knockdown. (A) Heat map of gene expression (mRNA) features representing sixteen canonical EMT markers of ZEB1 and/or WNT5A knockdown groups and miR-200 family or miR-205 over expression groups. Dark blue colors represent higher expression of that gene in that sample. These features were summarized into an EMT phenotype score for each sample. Dark purple colors represent a more mesenchymal-like phenotype. Samples are ordered by high to low average EMT score; (B) Histogram displaying EMT scores (mean ± SD) of negative control group, miRNA over-expression groups, and ZEB1 and/or WNT5A siRNA-knockdown groups. Values represent mean ± standard error of the mean (SEM). [statistically significant difference from negative control (* = $p < 0.05$; ** = $p < 0.01$); ns = not statistically significant]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

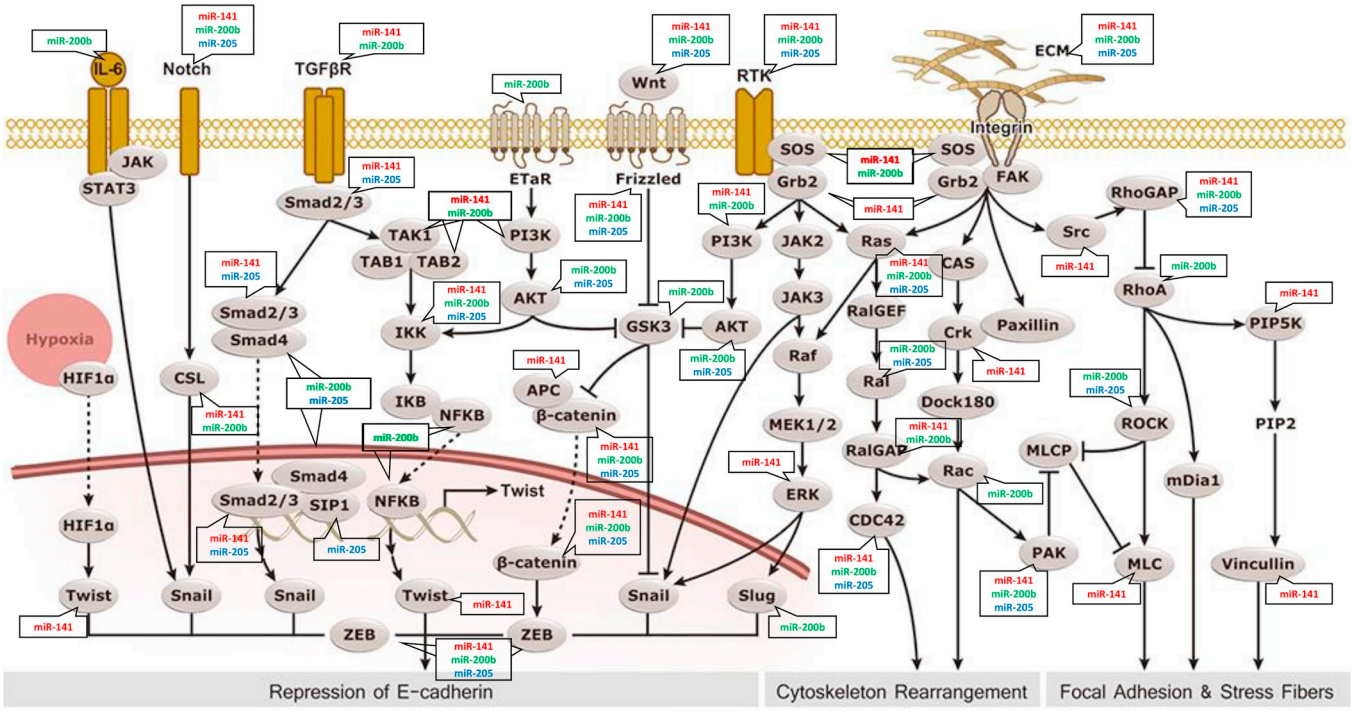

Fig. 7. MiR-200 family members (miR-141 and miR-200b) and miR-205 regulate key genes involved in EMT, invasion and metastasis pathways.

~80 MYA (estimated time of divergence of humans and mice from a common ancestor [8].

4. Discussion

In principle, the induction of similar morphological changes in response to over expression of the sequence divergent miR-200 family and miR-205 miRNAs may be explained by at least two not-mutually-exclusive hypotheses. First, if the miR-200 family and miR-205 miRNAs bind to the same mRNA (gene) target sequence(s), it could explain their coordinated regulation of the same genes. This hypothesis, however, is inconsistent with a large body of data indicating that high-sequence complementarity between miRNA seed regions and mRNA target sequence is pre-requisite to miRNA regulation [26]. Moreover, we have previously shown that even a single nucleotide substitution within the seed region of miR-200 family miRNAs is sufficient to dramatically disrupt downstream regulatory controls [5]. Thus, the high sequence divergence between miR-205 and members of the miR-200 family of miRNAs effectively precludes the possibility that they are targeting the same mRNA binding sites.

A second, more likely scenario is that many of the genes involved in the induction of MET in HEY cells contain duplicate yet distinct target sequences for miR-200 family and miR-205 miRNAs. Indeed, it is well documented that most mRNAs (genes) contain target sequences for more than one miRNA and are thereby subject to regulatory control by multiple miRNAs [26]. Although we find that only a small percentage (< 3%) of genes significantly differentially expressed in common after ectopic over expression of miR-205 or the miR-200 family of miRNAs (including genes previously implicated in EMT/MET) are predicted to be direct targets, changes in the expression of these direct targets are presumably the ultimate source of the subsequent regulatory changes, and many prior studies have confirmed the functional importance of these direct regulatory controls [5,26]. Among genes previously implicated in the EMT process that we found to be consistently down regulated after ectopic over expression of miR-205 or miR-200 family miRNAs in HEY cells, only *ZEB1* and *WNT5A* are predicted to be direct target genes of these miRNAs. While siRNA-mediated knock down of *ZEB1* and/or *WNT5A* was found to induce morphological and molecular changes characteristic of partial MET, it was not sufficient to recapitulate the more pronounced changes induced by the miRNAs. This finding is consistent with an auxiliary contribution of other direct and/or indirect regulatory changes induced in common and/or individually by miR-205, miR-200b and miR-141.

The fact that miR-205 targets fewer EMT/MET associated genes than members of the miR-200 family suggests that the contribution of miR-205 to the regulation of EMT/MET in humans may be a more recent evolutionary event. We previously reported that while the seed sequence of the miR-200 family of miRNAs is highly conserved across vertebrate species, the genes targeted by these miRNAs are highly diverse [5]. This observation coupled with the finding that only a single nucleotide substitution in the target sequence (seed region) of miRNA-regulated genes can result in loss/gain of regulatory controls, led to formulation of an evolutionary model whereby individual genes may lose and/or acquire new miRNA control(s) over time through relatively minor changes in miRNA target sequence [5]. Thus, one possible explanation of the more recent contribution of miR-205 to EMT/MET *trans*-regulation is that it may have more recently acquired an expression pattern compatible with the EMT/MET process in higher vertebrates, leading to subsequent selection for the acquisition of miR-205 target sequences in EMT/MET associated genes (*e.g.*, *WNT5A*, *ZEB1*). In this regard, it may be relevant to note that while miR-200b and miR-205 are clustered together on the same chromosome in humans (human chromosome 1) and display coordinated patterns of expression in at least partial response to the same regulatory controls [25], in the mouse, miR-205 maps to a distinct chromosomal location (mouse chromosome 1 [http://www.informatics.jax.org/marker/MGI:2676880]) from miR-200b (mouse chromosome 4 [http://www.informatics.jax.org/marker/MGI:2676875]) placing the two miRNAs under distinct regulatory controls.

5. Conclusion

MiR-205 and the miR-200 family of miRNAs coordinately induce MET in mesenchymal-like OC cells by affecting both direct and indirect

changes in the expression of genes previously associated with EMT/MET. While only two direct targets of these miRNAs (*ZEB*1 and *WNT5A*) are commonly down regulated in response to over expression of miR-205 and/or the miR-200 family of miRNAs, down regulation of these genes alone or in combination only partially recapitulates the changes induced by the miRNAs, indicating an auxiliary contribution of other direct and/or indirect regulatory changes induced in common or individually by the miRNAs. Our findings further suggest that regulation of EMT/MET associated genes by miR-205 may have been the result of convergent evolution facilitated by the relatively recent clustering of miR-205 with members of the miR-200 family on human chromosome 1–80 MYA. Collectively, our results point to the importance of indirect as well as direct regulatory changes induced by modulations in miRNA expression and the processes that drive acquisition in these regulatory controls over time. A full understanding of the indirect regulatory effects of miRNAs on global patterns of gene expression and the processes underlying acquisition of these regulatory controls will be essential to successful future clinical applications of miRNAs in both cancer diagnostics and therapeutics.

Conflicts of interest

All authors declare no conflict of interest.

Availability of data and materials

The microarray datasets supporting the conclusions of this article are available in the Gene Expression Omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE113970). Other datasets supporting the conclusions of this article are included within the article and its additional files.

CRediT authorship contribution statement

Mengnan Zhang: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing - review & editing. **Neda Jabbari:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology. **Minati Satpathy:** Investigation, Methodology. **Lilya V. Matyunina:** Investigation, Methodology. **Yuehua Wang:** Investigation, Methodology. **L. DeEtte McDonald:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. **John F. McDonald:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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List of abbreviations

CDH1	E-cadherin
EMT	Epithelial–to-mesenchymal transition
FN1	fibronectin
KRT7	keratin, type II cytoskeletal 7
KRT8	keratin, type II cytoskeletal 8
KRT18	keratin, type I cytoskeletal 18
MET	mesenchymal-to-epithelial transition
MiRNA	micro RNA
OC	ovarian cancer
qRT-PCR	quantitative real-time polymerase chain reaction
siRNA	small interfering RNA
VIM	vimentin
WNT5A	Wnt Family Member 5A
ZEB1	zinc finger E-box-binding homeobox 1
ZEB2	zinc finger E-box-binding homeobox 2

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.039.

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