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MicroRNA-106b-25 cluster targets β-TRCP2, increases the expression of Snail and enhances cell migration and invasion in H1299 (non small cell lung cancer) cells

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ABSTRACT

Lung cancer causes high mortality without a declining trend and non small cell lung cancer represents 85% of all pulmonary carcinomas. MicroRNAs (miRNAs) serve as fine regulators of proliferation, migration, invasion/metastasis and angiogenesis of normal and cancer cells. Using TargetScan6.2, we predicted that the ubiquitin ligase, β-TRCP2, could be a target for two of the constituent miRNAs of the miR-106b-25 cluster (miR-106b and miR-93). We generated a stable clone of miR-106b-25 cluster (CL) or the empty vector (EV) in H1299 (non small cell lung cancer) cells. The expression of β-TRCP2 mRNA was significantly lower in CL than that in EV cells. Transient expression of miR-93 but not antimiR-93 decreased the expression of β-TRCP2 mRNA in H1299 cells. β-TRCP2-3′UTR reporter assay revealed that its activity in CL cells was only 60% of that in EV cells. Snail protein expression was higher in CL than that in EV cells and H1299 cells exhibited an increase in the expression of Snail upon transient transfection with miR-93. miR-106b-25 cluster-induced migration of CL measured by scratch assay was more than that in EV cells and no significant difference in migration was observed between antimiR-93-transfected H1299 cells and the corresponding control-oligo-transfected cells. miR-106b-25 cluster-induced migration of CL cells was again confirmed in a Boyden chamber assay without the matrigel. CL cells were more invasive than EV cells when assessed using Boyden chambers with matrigel but there were no significant changes in the cell viabilities between EV and CL cells. Colony formation assay revealed that the CL cells formed more number of colonies than EV cells but they were smaller in size than those formed by EV cells. The supernatant from CL cells was more effective than that from EV cells in inducing tube formation in endothelial cells. Taken together, our data indicate that miR-106b-25 cluster may play an important role in the metastasis of human non-small cell lung cancer cells by directly suppressing the β-TRCP2 gene expression with a consequent increase in the expression of Snail.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. Non small cell lung cancer (NSCLC) represents 85% of all pulmonary carcinomas and the high mortality of NSCLC patients has not decreased significantly over the years [2]. Emerging evidences show that microRNAs (miRNAs) serve as fine regulators of proliferation, apoptosis, invasion/metastasis and angiogenesis of normal and cancer cells [3]. MicroRNAs (miRNAs) are 21–23 nucleotide-long, non-coding RNA molecules that recognize specific sites in the 3′untranslated region (3′UTR) of target mRNAs with perfect/imperfect base-pairing, ultimately leading to translational repression and/or mRNA degradation [4,5]. miRNAs can be used to sub-classify NSCLCs [6] and specific miRNA profiles may also predict prognosis and disease recurrence in early-stage NSCLCs [7,8]. miR-17-92 cluster is over expressed in lung cancers, particularly contributing to proliferation of small-cell lung cancer cells [9]. miR-17-92 cluster and its two paralogs (miR-106a-92 and miR-106b-25) have probably originated from a series of events during evolution including duplication and deletion [10]. Given their sequence similarities, these three miRNA clusters are predicted to have many highly overlapping targets [11]. The miR-106b-25 cluster is over expressed in a number of cancers and includes miRNA-106b (miR-106b), miRNA-93 (miR-93) and miRNA-25 (miR-25) [12,13]. miR-106b-25 cluster targets Smad7 and induces epithelial-to-mesenchymal transition in breast cancer cells [14]. Other targets of miR-106b-25 cluster include caspase-7 in prostate cancer [15] and IL8 in leiomyoma smooth muscle cells [16]. However, the role of miR-106b-25 cluster in NSCLC remains largely unexplored. In the present work we demonstrate that miR-106b-25

Abbreviations: β-TRCP, β-transducin repeat-containing protein; NSCLC, non small cell lung cancer; TGF-β, transforming growth factor-β; EV, pmiRex-EV (empty vector) expressing H1299 cells; CL, pmiRex-CL (miR-106b-25 cluster) expressing H1299 cells.

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cluster directly targets the 3′UTR of the β-TRCP2 transcript, increases the expression of Snail and promotes migration, colony formation and invasion of NSCLC cells and enhances tube formation in endothelial cells.

2. Materials and methods

2.1. Cell lines and maintenance

Human non small cell lung cancer cell line (H1299) was provided by Prof. H. Allgayer (DKFZ, Heidelberg, Germany) and EA.hy926 cells were obtained from Dr. C.J. Edgell (Tissue Culture Facility, UNC Lineberger Comprehensive Cancer Center, North Carolina, Chapel Hill, USA). HEK 293T cells were obtained from Dr. S. Mahalingam (Department of Biotechnology, IIT Madras, India) and all the cell lines were cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL) and antibiotics (Gibco BRL).

2.2. Plasmids and transfection

The pmiRex-EV [LemirR (Empty Vector)] and pmiRex-CL [miR-106b-25 cluster in LemirR] were provided by Prof. H.L. Ford (Program in Molecular Biology, University of Colorado, Aurora, USA). H1299 cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with 50 nM of hsa-miR-93 mimic (PM-93) or its inhibitor (AM-93) or negative control oligo (NC) purchased from Dharmacon (Lafayette Colorado, USA). β-TRCP2 3′UTR sequence was amplified from the genomic DNA of a healthy individual by polymerase chain reaction (PCR) and cloned into the *XbaI* site of the pGL3-control luciferase reporter (Promega, Madison, WI, USA) plasmid driven by the SV40 promoter. The primers (Sigma–Aldrich, Bangalore, India) used were: β-TRCP2 -3′UTR-S 5′CAATCTAGACAGTCTGCACTTT 3′ and β-TRCP2 -3′UTR-AS 5′CAATCTAGAGACTTGGACGG 3′. For reporter assay, EV or CL cells were co-transfected using lipofectamine 2000 (Invitrogen) with 1 μg of luciferase construct and 50 ng pRL-TK (Renilla Luciferase; Promega).

2.3. Generation of stable clones expressing miR-106b-25 cluster

6×10^6 HEK 293T cells were seeded in 10 cm dishes and cotransfected the next day with pmiRex-EV [LemirR (Empty Vector)] or pmiRex-CL [miR-106b-25 cluster in LemirR] along with helper plasmids (gag-pol and VSV-G). Medium with progeny virus from transfected HEK 293T cells was collected every 24 h for 2 days, and then filtered with 0.45-μm filters (Millipore) and concentrated using Vivaspin ultrafiltration spin columns (Sartorius). The viral fraction (50 μl) was used to transduce H1299 cells overnight in the presence of 8 μg/ml Polybrene (Sigma–Aldrich) in 6 well plates. Finally, transduced cells were treated with 2 μg/ml puromycin (Sigma–Aldrich) for 2 weeks for the selection of stable clones.

2.4. Western blotting

Western blot analysis was performed according to standard methods using anti-Snail antibody (Cell Signaling, Danvers, MA, USA) or anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA).

2.5. RNA extraction and quantitative real-time PCR

RNA fractions from EV or CL cells were isolated using Trizol (Invitrogen) according to the manufacturer's protocol. For quantitative real-time PCR (qPCR) of miRNA, 1 μg of the small RNA fraction was taken for cDNA synthesis in a 20 μl reaction. cDNA synthesis was carried out using stem-loop reverse transcription primers specific for miRNA-93-5p and similar primers for RNU6 served as internal control. 1 μl of this cDNA was used for qPCR using POWER SYBR green (Applied Biosystems, Foster City, CA, USA) and values were normalized to RNU6 levels. For qPCR of β-TRCP2 mRNA, 1ug of RNA was subjected to DNAse (Invitrogen) treatment and subsequently converted to cDNA using oligo dT primers for every 20 μl first strand synthesis reaction using MMLVRT (Invitrogen) and 1 μl of this cDNA was used for a 10 μl qPCR reaction. Expression values were calculated using the comparative Ct method and β-actin gene was used as endogenous control. Reactions were run in an Eppendorf realplex 4 Real-Time PCR System. The sequences of the sense (S) and antisense (AS) primers (Sigma–Aldrich, Bangalore, India) are as follows: β-TRCP2 – S, 5′ AAACCAGCCTGGAATGTTTG 3′, β-TRCP2 – AS 5′ CAGTCCATTGCTGAAGCGTA 3′, Actin – S 5′ TCATGAAGATCCTCACCGAG 3′, Actin – AS 5′ TTGCCAATGGTGATGACCTG 3′ hsa-miR-93-5p mature primer 5′ GCCCGCCAAAGTGCTGTTCGTGCA 3′, RNU6 mature primer 5′ CTGCGCAAGGATGACACGCA 3′, hsa-miR-93-5p stem loop primer 5′TCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTG 3′ and RNU6 stem loop primer 5′ GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTATGGAAC 3′.

2.6. Migration and invasion assay

The invasive and migration abilities of the cells were assessed in either matrigel (BD Biosciences) coated (10 μg matrigel/well) or uncoated Boyden chambers, respectively [17].

2.7. Luciferase reporter assay

Reporter assays were performed 48 h post-transfection as previously published [18] and readings were taken using a SpectramaxL luminometer (Molecular devices).

2.8. Cell proliferation assay

2000 EV or CL cells were seeded in 96-well plates in 100 μl 10% FCS growth medium. After 2, 24 or 48 h, growth inhibition was evaluated using 10 μl per well of CellTiter 96® AQueous One Solution (Promega). The absorbance at 490 nm was measured using an automated plate reader (Milenia Kinetic Analyzer).

2.9. Colony formation assay

EV or CL cells suspended in 0.6% agarose in DMEM were seeded in 6 well plates pre-coated with 0.3% agarose in DMEM in triplicates (2000 cells/well). DMEM was changed every 2 days. After 2 weeks, images were taken with 10× magnification.

2.10. Angiogenesis assay

In vitro angiogenesis assays were performed as previously described [19].

2.11. Statistical analysis

A two-tailed Student's *t*-test was used to evaluate the significance of the differences between two groups; p values <0.05 were considered significant.

3. Results

3.1. miR-106b-25 cluster targets β-TRCP2 with its miRNA response elements in the 3′UTR of β-TRCP2 and increases the expression of Snail

Genomic organization of polycistronic miR17-92 cluster and its paralogs (miR106a-92 and miR-106b-25) is shown in Fig. 1A. Using TargetScan6.2 we found that β-TRCP, known to be deregulated in NSCLC, is among the predicted targets for the constituent miRNAs of the polycistronic miR17-92 cluster and its paralogs. β-TRCP is a member of the ubiquitin ligase complex that ubiquitinates many proteins such as IκBα, WEE1, β-catenin and Snail [20]. β-TRCP1 is mainly present in the nucleus, whereas β-TRCP2 resides in the cytosol [20]. 3′UTR of β-TRCP2 (2585 nts) was screened for complementary seed sequences of known miRNAs and we found miR-17, miR-20a/b, miR-106a/b, miR-519d and miR-93 as potential microRNAs that can bind to an evolutionarily conserved site at nts 370-376 in the 3′UTR of β-TRCP2 (Fig. 1B). We first generated a stable clone of miR-106b-25 cluster in H1299 cells using pmiRex-CL (miR-106b-25 cluster). The CL clone exhibited 8 fold expression of miR-93 (one of the components of miR-106b-25 cluster) in comparison with the empty vector-transfected (EV) cells (Fig. 1C). Upon transient expression of PM-93, H1299 cells showed higher expression of miR93 (23 fold) than that in the control oligo-transfected (NC) cells (Fig. 1D). AM-93 (anti miR-93) expression efficiently down regulated the expression of endogenous miR-93 (Fig. 1E). To validate if β-TRCP2 was targeted by miR-106b-25 cluster, we analyzed the expression of β-TRCP2 mRNA in EV and CL cells and found that there was a significant decrease of β-TRCP2 mRNA in CL cells (Fig. 2A). Transient transfection of miR-93 decreased the expression of β-TRCP2 mRNA (Fig. 2B) but that of AM-93 slightly increased the expression of β-TRCP2 in H1299 cells (Fig. 2C). Prompted by these results we asked whether the 3′UTR of β-TRCP2 is a functional target of miR-106b-25 cluster and observed that β-TRCP2 3′UTR reporter activity was reduced to 60% in CL cells (Fig. 2D). These results suggest that β-TRCP2 is a target for at least one of the component miRNAs (miR-93) of miR-106b-25 cluster validating the predictions made from the Targetscan analysis.

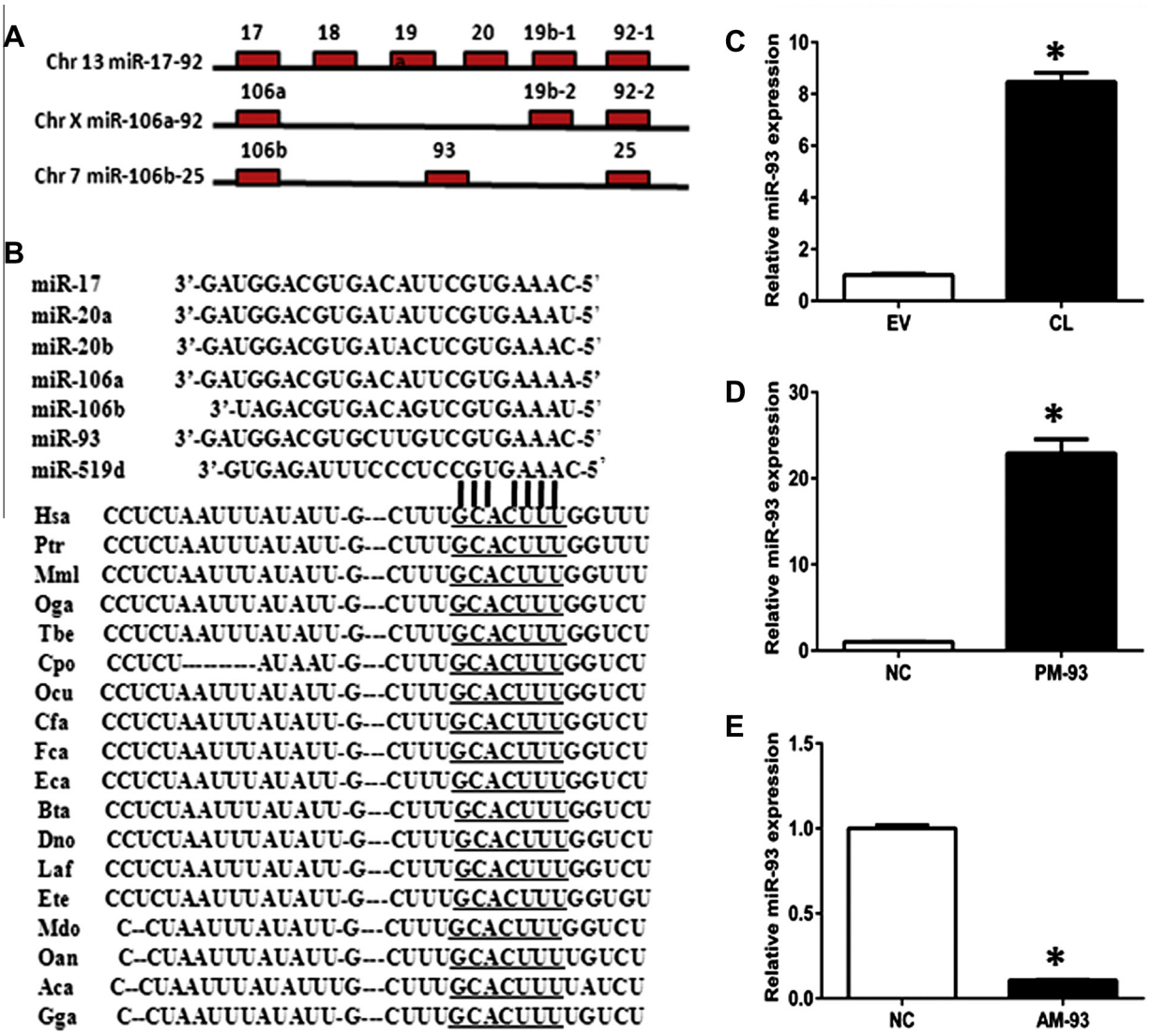

Fig. 1. Schematic representation of miR-17-92 cluster, its paralogs and miRNA response elements in β-TRCP2 mRNA and ectopic expression of miR-106b-25 cluster, PM-93 and AM-93 in H1299 cells. (A) Genomic organisation of miR17-92 cluster and its paralogs (miR106a-92 and miR-106b-25). (B) Seed sequences of miR17-92 cluster and its paralogs (miR106a-92 and miR-106b-25) and their conserved miRNA response elements in the 3′UTR of β-TRCP2. (C) EV and CL clones were analysed for the expression of miR-93 by q-PCR. (D) H1299 cells transiently transfected with PM-93 or NC were analysed for the expression of miR-93 by q-PCR after 48 h. (E) H1299 cells transiently transfected with AM-93 or NC were analysed for the expression of miR-93 by q-PCR after 48 h as described in Section 2. The experiments described above were repeated three times and the error bars denote the mean ± S.E. (*$p < 0.05$).

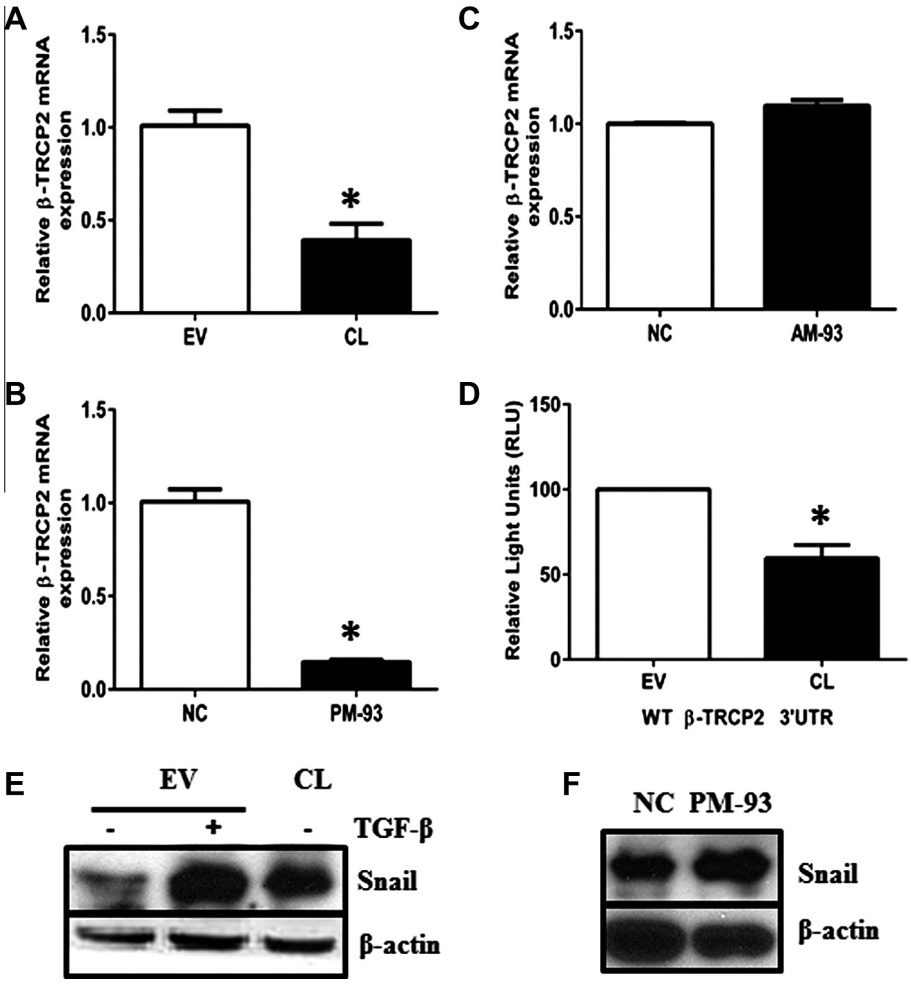

Fig. 2. Effects of ectopic expression of miR-106b-25 cluster, PM-93 and AM-93 on the expression of β-TRCP2 mRNA and Snail in H1299 cells. (A) EV and CL cells were analysed for the expression of β-TRCP2 mRNA by q-PCR. (B) H1299 cells transiently transfected with NC or PM-93 were analysed for the expression of β-TRCP2 mRNA after 48 h by q-PCR. (C) H1299 cells transiently transfected with NC or AM-93 were analysed for the expression of β-TRCP2 after 48 h by q-PCR as described in Section 2. (D) EV and CL cells transiently co-transfected with pRL-TK and wild type (WT) 3′UTR of β-TRCP2, were analyzed for the luciferase activity after 48 h. The experiments described above were repeated three times and the error bars denote the mean ± S.E. ($^*p < 0.05$). (E) EV cells were treated for 48 h with TGF-β (10 ng/ml) that served as a positive control. EV or CL cells were analyzed by western blotting for the expression of Snail and β-actin as described in Section 2. (F) H1299 cells transiently transfected with NC or PM-93 were similarly analysed for the expression of Snail and β-actin by western blotting. Results from all the above experiments were confirmed in at least two independent experiments.

Since β-TRCP2 is known to degrade Snail [20], it was of interest to analyse the effect of miR-106b-25 cluster on the expression of Snail. Snail protein expression was higher in CL than that in EV cells suggesting that the miR-106b-25 cluster up regulates the expression of Snail (Fig. 2E). TGF-β was used as a positive control as it is known to increase the expression of Snail [21] and as expected it enhanced the expression of Snail in EV cells (Fig. 2E). Transient transfection of PM-93 also increased the expression of Snail in H1299 cells (Fig. 2F). These results suggest that miR-106b-25 cluster or at least one of its components clearly targets β-TRCP2 and concurrently up regulates the expression of Snail.

3.2. miR-106b-25 cluster promotes in vitro migration, invasion and colony formation, of H1299 cells and enhances tube formation in endothelial cells

As Snail is known to be involved in the process of migration and invasion [21,22], we performed scratch and transwell migration assays first in EV and CL cells. miR-106b-25 cluster-induced migration of CL cells was more than that in EV cells within 8 h and the wound closure was complete in 24 h in CL but not in EV cells (Fig. 3A and C). Boyden chamber assays without the matrigel also confirmed that the CL cells migrate better than the EV cells (Fig. 3E). To further investigate the specific role of miR-93 in wound closure, we performed the scratch assay in AM-93-transfected H1299 cells and found no significant difference in migration as compared to control (NC) cells (Fig. 3B and D). Although miR-106b-25 cluster targets β-TRCP2, there were no significant changes in the cell viabilities of EV and CL cells up to 48 h (Fig. 4A). However, CL cells were more invasive than EV cells when assessed using Boyden chambers with matrigel (Fig. 4B). Cancer cells acquire anoikis resistance to survive after detachment from the primary sites and travel through the circulatory and lymphatic systems to disseminate throughout the body [23]. To assess the effect of miR-106b-25 cluster on such anchorage independent tendencies, colony formation assay was next performed. The results reveal that the miR-106b-25 cluster induced more number of colonies although their size was smaller than those formed by EV cells (Fig. 4C) suggesting that the EV cells could have undergone differentiation while CL cells acquired tumorigenic properties. Endothelial cells represent one of the critical cellular components in tumor microenvironment with a role in the growth and progression of

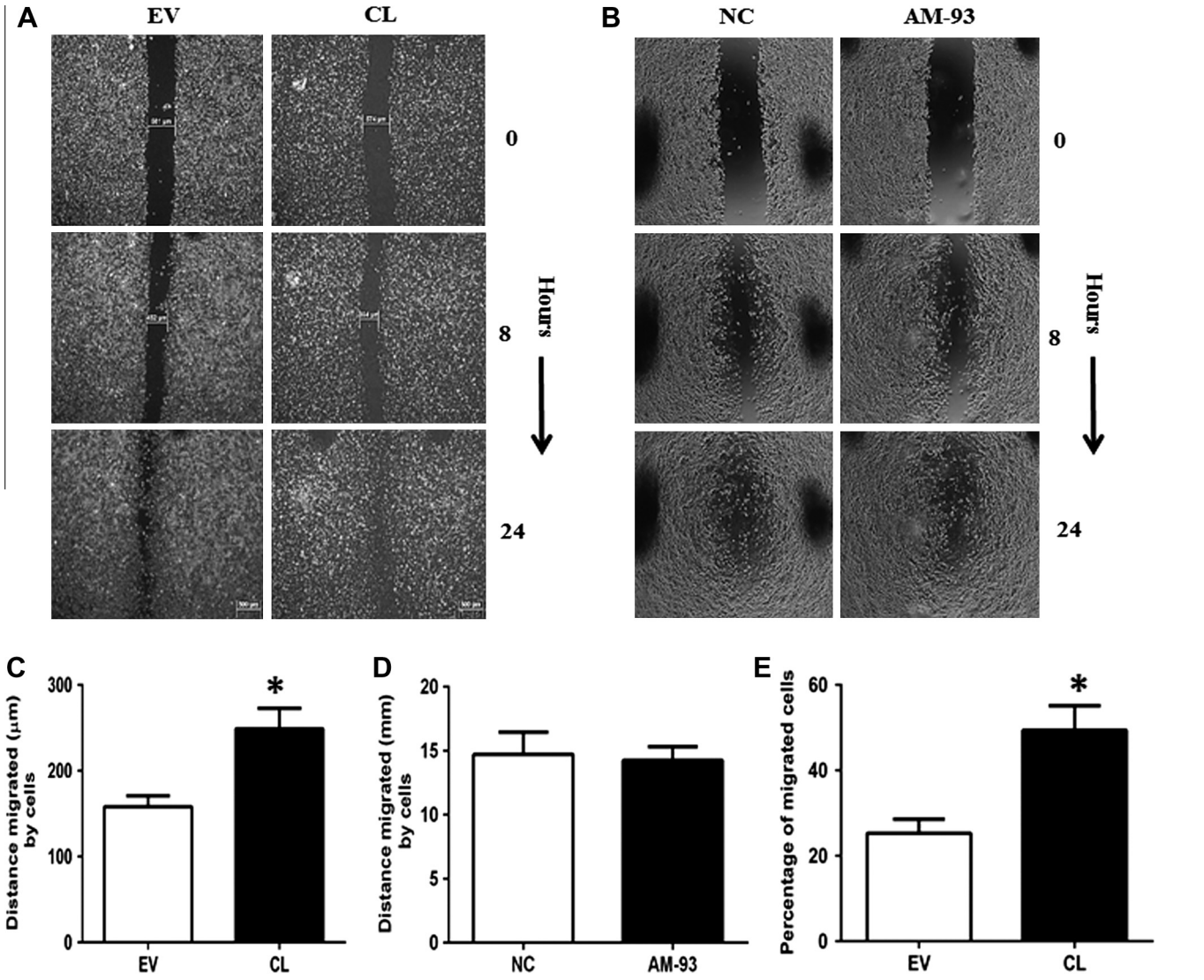

Fig. 3. Effects of ectopic expression of miR-106b-25 cluster on migration of H1299 cells. (A) Scratches were made in confluent EV and CL clones with a 100 μl pipet tip and the images (4× magnification) were taken at the indicated time intervals from a representative experiment. (B) H1299 cells were transiently transfected with NC or AM-93 and scratches were made after 24 h in confluent cells with a 100 μl pipet tip and the images (10× magnification) were taken from a representative experiment. (C and D) The distance migrated by the cells under the same conditions described above was quantitated. The experiment was repeated three times in triplicates and the error bars denote the mean ± S.E. (*$p < 0.05$). (E) EV or CL clones were plated on the top of the Transwell Boyden chambers and the migrated cells were measured after 24 h as described in Section 2. Percentage of migrating cells is shown as mean ± S.D. of four replicates (*$p < 0.05$).

cancer by controlling angiogenesis. Towards this end, EA.hy926, a cell line derived from human umbilical vein endothelial cells that expresses a highly differentiated functional characteristic of human vascular endothelium [24–27], was used to assess the role of miR-106b-25 cluster in angiogenesis. The supernatants from EV and CL cells were tested for their ability to induce tube formation in EA.hy926 cells. The results indicate that the supernatant from CL cells (CL sup) was more effective than that from EV cells (EV sup) in inducing tube formation in endothelial cells (Fig. 4D). These data suggest that miR-106b-25 cluster does not affect the viability of H1299 cells but increases their migration and invasive potential. They also suggest that the cluster induced colony formation in H1299 cells and favored tube formation in endothelial cells.

4. Discussion

The present work is focused on understanding the role of miR-106b-25 cluster in NSCLCs by experimentally validating one of its predicted targets, β-TRCP2. Our data showing a decrease in the expression of β-TRCP2 by the miR-106b-25 cluster in H1299 cells are novel and loss of β-TRCP1 has been shown to enhance both growth and cell motility of lung cancer cells [28]. Decreased expression of β-TRCP2 by the ectopic expression of miR-106b-25 cluster in H1299 cells with a concomitant increase in the expression of Snail is consistent with the proposed role of β-TRCP2 in the degradation of Snail [20]. However, H1299 cells lack E-cadherin [29] and its expression is known to be repressed by Snail [30]. Interestingly, Snail is a target of miR-30 and the reduction in its expression by this miRNA reduces the migration, invasion and metastasis of H1299 cells [22]. Human NSCLC tissues contain high levels of Snail that correlate with decreased survival, and Snail overexpression is associated with increased angiogenesis and enhanced tumor growth *in vivo* [31]. Snail also regulates cell adhesion and migration [32,33]. Snail-expressing cells exhibit migratory and invasive properties [34], and impairment in cell division [35].

Although miR-106b-25 cluster does not affect the proliferation of H1299 cells, miR-106b promotes cell proliferation in laryngeal carcinoma [36]. miR-93 suppresses proliferation of human colon

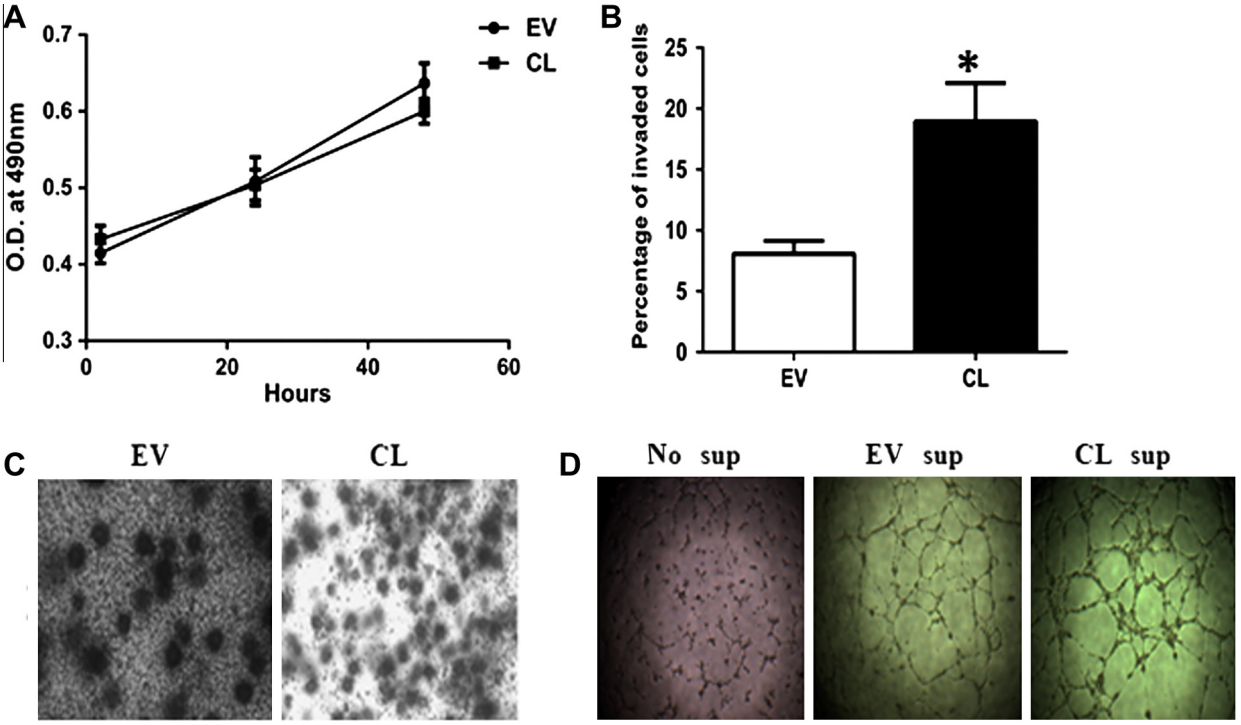

Fig. 4. Effects of ectopic expression of miR-106b-25 cluster on viability, invasion, anchorage-independent growth and tube formation. (A) EV and CL cells were analysed for cell viability as described in Section 2. The experiment was repeated three times and the error bars denote the mean ± S.E. (B) EV or CL clones were plated on the top of the matrigel-coated Boyden chambers and the invaded cells were measured as described in Section 2. Data are represented as the percentage of invading cells, as mean ± S.D. of four replicates ($^*p < 0.05$). (C) EV or CL clones suspended in 0.6% agarose in DMEM were seeded in 6 well plates pre-coated with 0.3% agarose in DMEM in triplicates (2000 cells/well) and after 2 weeks, images were taken with 10× magnification. (D) Supernatants from EV or CL clones were added to Ea.hy926 endothelial cells grown in matrigel-coated 96-well plates and after 16 h images were taken with 4× magnification. The tube formation was analysed as described in Section 2. Similar results were obtained in another independent experiment.

cancer stem cells [37], but enhances proliferation in HepG2 [38] and osteosarcoma cells [39]. miR-25 promotes cell migration and invasion in oesophageal squamous cell carcinoma [40], and mir-93 enhances migration of HepG2 cells [38]. Interestingly, β-TRCP-depleted endothelial cells exhibit enhanced migration and angiogenesis *in vitro* [41]. miR-93 enhances cell survival, supports endothelial cell spreading, growth, migration and tube formation, resulting in enhanced tumor growth of glioblastoma cells [42]. Expression of miR-93 is high in squamous-cell carcinoma subtypes of NSCLC patients with shorter overall survival [43,44].

Our results highlight the loss of β-TRCP2 and increased expression of Snail in H1299 cells upon ectopic expression of miR-106b-25 cluster suggesting that these conditions may favor migration and invasion. Taken together, our data indicate that miR-106b-25 cluster may play an important role in the metastasis of human NSCLC cells by directly suppressing the β-TRCP2 gene expression with a consequent increase in the expression of Snail. Further work is in progress to unravel the role of the component miRNAs of miR-106b-25 cluster and confirm the results *in vivo* using suitable animal models.

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