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Molecules and Cells

Aberrant microRNAs Expression in CD133$^+$/CD326$^+$ Human Lung Adenocarcinoma Initiating Cells from A549

Sheng Lin[1,2,3], Jian-guo Sun[1,3], Jing-bo Wu[2], Hai-xia Long[1], Cong-hui Zhu[1], Tong Xiang[1], Hu Ma[1], Zhong-quan Zhao[1], Quan Yao[1], An-mei Zhang[1], Bo Zhu[1,*], and Zheng-tang Chen[1,*]

Increasing evidence demonstrates that miRNAs are involved in the dysregulation of tumor initiating cells (TICs) in various tumors. Due to a lack of definitive markers, cell sorting is not an ideal separation method for lung adenocarcinoma initiating cells. In this study, we combined paclitaxel with serum-free medium cultivation (inverse-induction) to enrich TICs from A549 cells, marked by CD133/CD326, defined features of stemness. We next investigated aberrant microRNAs in this subpopulation compared to normal cells with miRNA microarray and found that 50 miRNAs exhibited a greater than 2-fold change in expression. As further validation, 10 miRNAs were chosen to perform quantitative RT-PCR on the A549 cell line and primary samples. The results suggest that aberrant expression of miRNAs such as miR-29ab, miR-183, miR-17-5p and miR-127-3P may play an important role in regulating the bio-behavior of TICs.

INTRODUCTION

Lung cancer ranks highly as a leading cause of tumor mortality, with about more than 1 million deaths per year worldwide. In recent decades, the survival of patients with has improved remarkable due to modifications in diagnosis, surgery and combined modality therapy. However, the overall 5-year survival rate has changed little; most patients die of recurrence or metastasis (Peacock et al., 2008).

Increasing evidence has revealed that primary tumors contain a rare subpopulation, termed cancer stem cells (CSCs) or tumor initiating cells (TICs), responsible for maintaining tumor growth, possessing stronger capacity of self-renewal, invasion and therapy-resistance, resulting in recurrence and metastasis. The existence of CSCs was first verified by Bonnet et al. (1997): they isolated a CD34$^+$/CD38$^-$ subpopulation from acute myeloid leukemia and confirmed that these cells had features of normal stem cells. CSCs were then described by some putative cell surface markers and reported in various solid tumors, including breast (CD44$^+$/ CD24$^{low/-}$, Al-Hajj et al., 2003), melanoma (CD20$^+$ or CD133$^+$, Zabierowski et al., 2008), and brain (Singh et al., 2004), prostate (Collins et al., 2006), ovary (Zhang et al., 2008), liver (Ma et al., 2010), pancreas (Hermann et al., 2007) and colon (Ricci-Vitiani et al., 2007), all of which express CD133$^+$. However, the markers of lung CSCs remain controversial. Extensive study of spheres and tumorigenesis showed that the CD133$^+$ subpopulation had more stem-like properties than its CD133$^-$ counterpart from primary specimens (Eramo et al., 2008). *In vivo* and *in vitro* experiments demonstrated that the CD133$^+$/CD326$^+$ or CD34$^+$/CD326$^+$ subpopulations represent CSCs in primary tumors, but in cell lines including A549, CALU1, LC12, LC31 and LC52, only the CD133$^+$/CD326$^+$ subpopulation possessed stemness (Tirino et al., 2009).

MicroRNAs (miRNAs) are a class of small, endogenous, non-coding, 19-23 nucleotide RNAs that negatively regulate gene expression by partial or entire complementary binding to 3′ UTR of mRNAs, resulting in either posttranscriptional represssion or RNA degradation. Several studies reveal that aberrant expression of miRNAs is involved in human disease, including cancers. Some miRNA expression profiles of cancer patients show correlation with the stage, progression and prognosis, suggesting that miRNAs can serve as oncogenes or tumor suppressors involved in regulating tumor progression (Jiang et al., 2008; Ura et al., 2009; Wiemer et al., 2007). Emerging evidence reveals that abnormal miRNA expression is relevant to the dysregulation of CSCs in various cancers. Elevated miR-181 clusters were identified as vital regulators in EPCAM$^+$ hepatic tumor initiating cells (Ji et al., 2009). Down-regulation of miR-200 clusters in breast cancer stem cells and normal stem cells indicted a common molecular mechanism of stem cell functions (Shimono et al., 2009). Up-regulation of miR-128 in glioma stem cells showed a significant reduction of self-renewal by targeting Bim-1 mRNA, suggesting that miR-128 may be a potential therapeutic target of glioma stem cells (Godlewski1 et al., 2008). *In vivo* and *in vitro* studies indicated that up-regulation of miR-199b-5p impaired the development of CSCs though repression of HES1 in medulloblastoma (Garzia et al., 2009). Restoration of miR-34 expression clearly repressed the self-renewal of CSCs in pancreatic cancer (Ji et al., 2009).

[1]Institute of Cancer, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China, [2]Oncology Department, The Affiliated Hospital of Luzhou Medical College, Sichuan 646000, China, [3]These authors contributed equally to this work.
*Correspondence: zhengtangchen@126.com (ZTC); lslinxiaosheng@126.com (BZ)

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In this study, we combined inverse-induction with paclitaxel treatment to select CSCs from the A549 cell line and identified that the enriched cells marked by $CD133^+$/ $CD326^+$ possessed stemness. Meanwhile, we found that $CD133^+$/ $CD326^+$ cells reside in fresh tumor samples. Next, we performed microarray analysis on this subpopulation compared to the normal cancer cells and quantitative RT-PCR on samples both cell line and primary tumors to validate the array data. From our data, we hope to establish a systemic recognition of aberrant miRNAs in lung adenocarcinoma initiating cells and partly reveal the underlying mechanism between CSCs and stem cell miRNAs.

MATERIALS AND METHODS

Inverse-induction and paclitaxel treatment to isolate $CD133^+/CD326^+$ cells from the A549 cell line

A549 cells were obtained from the American Type Culture Collection. After dissociation with trypsin (Invitrogen), approximately 10^6/mL cells were suspended in serum-free medium supplemented with 0.4% BSA (Sigma), insulin (5 μg/ml, Sigma), basic fibroblast growth factor (bFGF, 10 ng/ml, PeproTech), human recombinant epidermal growth factor (EGF, 20 ng/ml, PeproTech). Spheres were mechanically dissociated into single cells or small aggregates to expand in serum-free medium. At the second passage, paclitaxel injection (30 mg/5 ml, Powerdone, China) was added at a concentration of 100 nmol/L for 48 h and then replaced with completely fresh medium once or twice per week until new spheroids emerged.

Flow cytometry analysis

Spheroids were dissociated into single cells, washed and incubated with monoclonal antibodies specific for human PE-conjugated CD133/1, FITC-conjugated Ep-CAM (CD326, Miltenyi Biotec). The appropriate dilution and procedures were carried out according to the manufacturer's instructions. After incubation for 30 minutes, cells were washed again and analyzed by flow cytometry.

Immunofluorescence

Spheroids were centrifuged onto slides by cytospin, fixed with 4% paraformaldehyde for 20 min and blocked with normal serum for 30 min at room temperature. Slides were then incubated with rabbit monoclonal anti-CD133 (Abcam) and goat polyclonal anti-EP-CAM (Santa Cruz) at dilution of 1:300 and stored at 4°C overnight protected from light. After washing, slides were incubated with FITC-conjugated goat anti-rabbit IgG (Beyotime) and Cy3-conjugated donkey anti-goat IgG (Biolegend) fluorescent antibodies at dilution of 1:400 for 30 min. After DAPI staining for nuclei, slides were examined by an Olympus confocal microscope. Immunofluorescence on tumor tissue sections was according to above procedures except for the dilution of antibodies (primary and secondary antibodies were 1:200 and 1:300, respectively).

Immunohistochemistry on differentiation of stem cell progeny

Spheres (4^{th} generation) were centrifuged onto coverslipes, placed in a 6-well plate and resuspended in media with 10% FBS without growth factors. At the sixth day of culture, cell morphology was examined by phase-contrast microscope. The coverslipes containing a confluent cell layer were washed with PBS and fixed with 4% paraformaldehyde. After treatment with 0.3% H_2O_2 for 20 min, the coverslipes were stained with rabbit monoclonal anti-CK-8/18 (Zhongshan Goldenbridge Biotechnology Company, China) at 4°C overnight. After washing, the coverslipes were incubated with goat anti-rabbit IgG (Zhongshan Goldenbridge Biotechnology Company, China) at 37°C for 30 min. After haematoxylin counterstaining with DAB (DAKO) and gradient-dehydration by ethanol, the coverslipes were fixed with resin and submitted for examination.

Magnetic activated cell sorting (MACS) of CSCs from primary lung adenocarcinoma

Tumor tissues were from the department of thoracic surgery, Da ping Hospital, Third Military Medical University, China. Tumor samples were washed several times by PBS supplemented with a high dose of penicillin/streptomycin and cut into nubbles. After incubation with collagenase I (2 mg/ml) at 37°C for 1 h, tumor tissues were filtered into single cell suspensions by a 30 μm cell strainer. According to the manufacturer's instructions, 1×10^8 cells/300 μl were incubated with 100 μl CD133/1 (AC133, mouse IgG, cell isolation kit, Miltenyi) microbeads and and 100 μl FcR blocking buffer for 25 min on ice. Cells were then sorted by the MACS separator (Miltenyi Biotec). The efficiency of sorting was verified by flow cytometry with PE-conjugated-CD133/2 antibody (Miltenyi Biotec).

Generation of subcutaneous xenografts on nude mice

Four-week-old male nude mice were purchased from the Chinese Academy of Medical Sciences. To generate xenografts, $CD133^+/CD326^+$ cells were injected into the left armpit of the forelimb while A549 normal cancer cells were injected into the right armpit. Serial dilutions of $CD133^+/CD326^+$ cells were 1×10^5, 1×10^4 and 5×10^3 while those of the normal cancer cells were 1×10^5, 1×10^4 and 2×10^6. Mice were observed twice a week and sacrificed until tumors reached approximately 2 cm at maximum diameter.

MicroRNA microarray and data analysis

Total RNA was isolated with Trizol reagent. The following protocols, including quality control, labeling (FlashTag™ Biotin), hybridization (Affymetrix® GeneChip® miRNA Arrays) and scanning were performed by CapitalBio (http://www.capitalbio.com). The probes covered the most up-to-date in the Sanger miRBase V11 (http://microrna.sanger.ac.uk). SnoRNAs and scaRNAs were derived from the snoRNABase (www.snorna.biotoul.fr/coordinates.php) and the Ensembl Archive (www.ensembl.org/ biomart/martview). Comparison of the two samples was based on the Wilcoxon Rank-Sum test. MiRNAs displaying more than a 2-fold change in expression were considered significant. The major instruments included the Affymetrix® Hybridization Oven 640, the Affymetrix® Fluidics Station 450 and the Affymetrix® GeneChip® Scanner 3000.

RT-PCR and quantitative RT-PCR

To confirm the array data, 10 miRNAs were chosen for quantitative RT-PCR. Reverse transcription reactions were performed using M-MLV Reverse Transcriptase (Invitrogen) with RNase inhibitor (Promega) and miRNA specific stem-loop RT-Primer (Invitrogen) to made cDNA from total RNA. The parameters of the RT reaction incubations were: 16°C for 10 min, 37°C for 30 min and then 65°C for 5 min. The parameters of PCR were: 95°C for 10 min followed by 40 cycles, 95°C for 15 s and 60°C for 1 min. Melting curves of each reaction were analyzed to remove any nonspecific amplification. The miRNAs expression was calculated using the Ct method and normalized by U6 gene expression. Key instruments included the Peltier Thermal Cycler PTC-225 (M J), 7900 HT Fast RealTime PCR system (Applied Biosystems). CSCs associated genes expression were measured and quantified by the manufacturer's instruc-

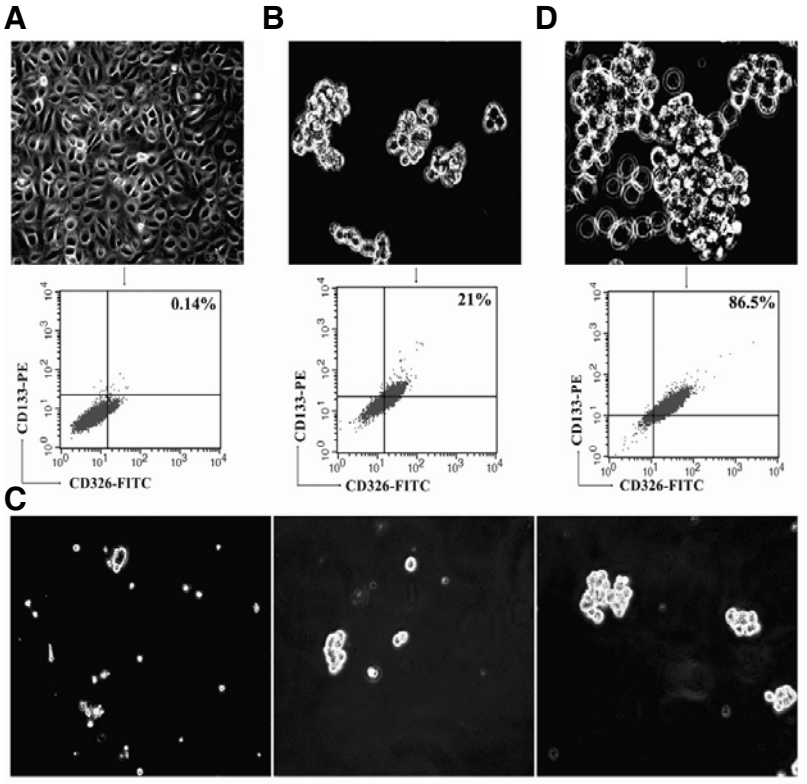

Fig. 1. Separation of the CD133$^+$/CD326$^+$ subpopulation by inverse-induction combined with paclitaxel treatment from A549 cells. (A) Cytometric CD133/CD326 expression in normal A549 cells, (B) secondary passage spheroids induced by single inverse-induction, (C) the progression of regrowing into spheres after treatment with paclitaxel (from left to right, showed the morphology on the 4th day, 10th day and 14th day, 200×) (D) Cytometric CD133/CD326 expression in secondary passage spheroids after paclitaxel treatment.

tions using the SYBR®Premix Ex Taq™ Kit (Takara Dalian, China). Each sample was done in triplicate and the reaction products were analyzed by the ABI 7300 Prism Sequence Detection System (Applied Biosystems, USA), using the following reactions: 95°C for 30 s, 35 cycles of 95°C for 5 s, 60°C for 34 s and 72°C for 40 s. Each experiment was controlled with GAPDH as a reference standard. Data analysis was based on the Ct method ($\Delta\Delta$Ct according to Applied Biosystems, the primers are shown in Supplementary Table S1).

Bioinformatic analysis of target gene prediction

Three online programs including PicTar, miRanda and TargetScan were used to predict the potential target genes of selected miRNAs.

RESULTS

Inverse-induction combination with paclitaxel induces a CD133$^+$/CD326$^+$ subpopulation from the A549 cell line

Normal A549 cells were resuspended in serum-free medium and grown into spheres. At the second passage, paclitaxel (100 nmol/L) was added into the culture for 48 h and then replaced with fresh medium. After paclitaxel treatment, most cells were clearaged. During a period of approximately two weeks, the surviving cells were able to gradually regrow into spheres (Fig. 1C). Because CD133$^+$/CD326$^+$ cells could represent CSCs derived from A549 (Tirino et al., 2009), we tried these two markers on different samples by flow cytometry. We found that the expression of CD133 and CD326 was approximately 0.2-0.5% on normal A549 cells (Fig. 1A), approximately 20% on secondary spheroids after single inverse-induction (Fig. 1B) and more than 80% on spheroids after paclitaxel treatment (Fig. 1D). These data suggest that the subpopulation of cells that survived inverse-induction and paclitaxel treatment could highly express the CD133/CD326 molecular markers.

Identification of CD133$^+$/CD326$^+$ cells as TICs of A549

To further verify whether the CD133$^+$/CD326$^+$ cell subpopulation possessed the traits of tumor initiating cells, we carried out some experiments *in vitro* and *in vivo*. At the 4th sphere progeny, immunofluorescence demonstrated that most of the spheroids were positive for CD133 and CD326 (Fig. 2A). Under differentiating conditions, the spheroid-derived cells adhered to plates after 24 h and their morphology was similar to that of normal A549 cells on day 6. Almost all cells expressed CK8 or CK18 by immunohistochemistry which showed that this subpopulation was capable of differentiation (Fig. 2B). *In vivo* subcutaneous xenografts on nude mice showed that just as few as 1×10^4/ml CD133$^+$/CD326$^+$ cells could generate xenografts while 1×10^5/ml normal cells failed to do so. The histologic examination showed similar results with adenocarcinoma (Fig. 2C and Table 1). Furthermore, stem cell-associated genes, including CD133, CD326, OCT4, Nanog and Nestin, were detected by qRT-PCR and all showed higher expression in CD133$^+$/CD326$^+$ cells compared to normal cancer cells (Fig. 2D). These data demonstrate that CD133$^+$ /CD326$^+$ cells have features of stemness including self-renewal, differentiation, high expression of CSCs-associated genes and stronger capability of tumorigenesis.

CD133$^+$/CD326$^+$ cells co-localize in primary lung adenocarcinoma

As CD133$^+$/CD326$^+$cells can be considered TICs of A549, we wondered if this subpopulation resides in the primary lung adenocarcinoma samples. As expected, we found CD133$^+$/CD326$^+$ cells expressed in the same area in fresh surgical sections (Fig. 3A) and the postoperative tumor samples were validated to be

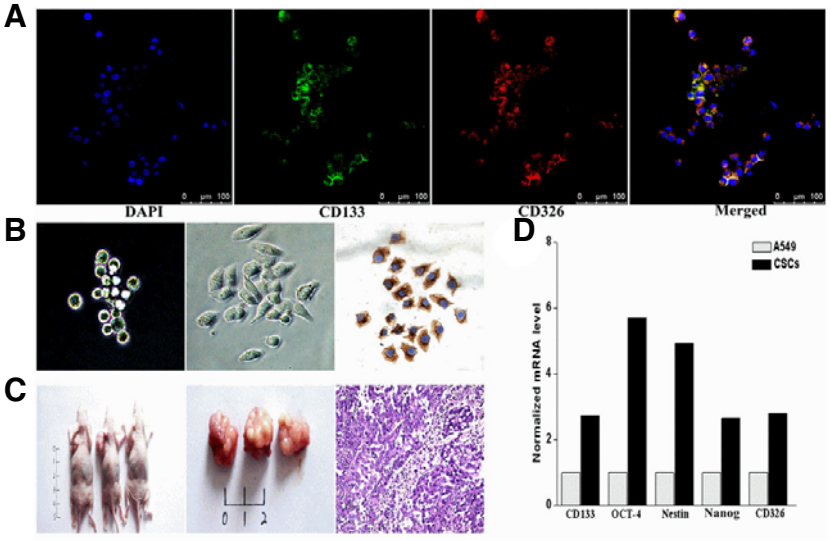

Fig. 2. Identification of the CD133+/CD326+ subpopulation as CSCs of A549. (A) Immunofluorescence of CD133 (green) and CD326 (red) expression on spheroids after paclitaxel treatment. (B) Differentiation of spheroids into progeny upon conditioning with complete medium. From left to right: morphology change on the second day, sixth day and expression of CK8/CK18 on progeny (200×). (C) Subcutaneous xenografts on nude mice (left armpit of forelimb, 1×10^4 CD133+/CD326+ cells; right armpit of forelimb, 1×10^4 normal A549 cells), all mice were sacrificed after injection for 3 weeks and observed by histology (200×). (D) Detection of stem cell-associated genes between the CD133+/CD326+ cells and normal A549 cells (defined as 1) by quantitative RT-PCR.

Table 1. Subcutaneous xenografts on nude mice (male, 4-week-old)

Cell number	2×10^6	1×10^5	1×10^4	5×10^3
CD133+/CD326+ cells		3/3	3/3	0/3
A549 normal cells	3/3	0/3	0/3	
Latency (days)	13	15	15	

CD133+/CD326+ cells were injected into the left armpit of forelimb at dilutions of 1×10^5, 1×10^4 and 5×10^3 while 1×10^5, 1×10^4 and 2×10^6 of normal A549 cells were injected into the right counterpart. All mice were sacrificed when either tumor reached approximately 2 cm at maximum diameter.

adenocarcinoma by histology (Fig. 3B). Eramo et al. (2008) reported that the CD133+ marker could represent TICs in primary lung cancers. Using MACS, we successfully isolated the CD133+ positive cells from primary samples and confirmed CD133 by flow cytometry. Expression of CD133/CD326 before sorting and after sorting was 1.2% and 93%, respectively (Fig. 3C). The cells of before sorting and after sorting were cultured with respective manner, their morphology was shown in (Fig. 3D).

Aberrant miRNA expression in CD133+/CD326+ cells

Consistent with the important regulatory roles of microRNAs, we performed a microarray to screen for aberrant miRNAs in the CD133+/CD326+ subpopulation and found that 14 miRNAs were up-regulated and 36 miRNAs were down-regulated compared to normal A549 cells (Fig. 4A; array data are shown in Supplementary Fig. S1 and the full list of miRNAs is shown in Supplementary Table S2). While considering the major traits of TICs, we chose 10 miRNAs for qRT-PCR analysis that might be involved in tumorigenesis, invasion, metastasis, therapy-resistant and epithelial-mesenchymal transition (EMT) including up-regulated miR-663, miR-183, miR-125a-5p, miR-127-3p and miR-520h, down-regulated miR-18b, miR-29a, miR-29b, miR-17 and miR-155. The expression trends of miR-29a, miR-29b, miR-155, miR-183, miR-127-3p, miR-17 were consistent with the array data (Fig. 4B).Moreover, to narrow the future research range and enhance the clinical relevance, we validated these miRNAs in primary samples and found the expression trend of miR-183, miR-127-3p, miR-17 were consistent with the results of the microarray at the cell line level (Figs. 4C and 4D).

Preliminary bioinformatic analysis of miRNAs associated with CD133+/CD326+ cells

Using online programs, we found that most of the potential target genes are oncogenes, tumor suppressors or regulators of transcription, signal transduction, apoptosis, cell cycle and invasion. For example, potential target genes of miR-183 include ZEB1 (Wellner et al., 2009), EGR1 (Saver et al., 2010), PTEN (Saver et al., 2010), VIL2 (Wang et al., 2008), PDCD4 (Li et al., 2011), ITGB1 and KIF2A (Li et al., 2010), etc. Potential target genes of miR-17 include A1B1 (Hossain et al., 2006), E2F1 (Tagawa et al., 2007), CCND1 (Cloonan et al., 2008), STAT3 (Zhang et al., 2011), RBI2 (Lu et al., 2007), HBP1 (Li et al., 2011), etc. Potential target genes of miR-127 include BCL6 (Saito et al., 2006), E2F3 and NOTCH1 (Tryndyak et al., 2009), etc. These genes are involved in controlling cellular proliferation, angiogenesis, cell cycle, transformation and metastasis, and play important roles in tumorigenesis and progression (Supplementary Table S3).

DISCUSSION

CD133, a putative molecular marker, is commonly used for MACS or FACS to separate CSCs from various tumor types. However, in non-small cell lung cancer, using CD133 as a marker remains controversial. Meng et al. (2009) demonstrated that both CD133+ and CD133- subpopulations contained similar numbers of TICs, suggesting CD133 alone could not be a TICs marker, at least in the A549 and H446 cell lines. Tirino et al. (2009) reported that in stable cell lines, including A549, only the CD133+/CD326+ subpopulation could represent TICs. Other studies reported that inverse-induction was a method to enrich TICs from oral squamous cell carcinoma (Chiou et al., 2008). Based on drug resistance, surviving cells could be regarded as TICs (Bertolinia et al., 2009; Levina et al., 2008; Teng et al., 2010)

Considering the above reasons, we first decided to obtain TICs from A549 cells by inverse-induction. However, we found the expression of CD133+ could not exceed 45%, even when the cells were continuously expanded to the10th generations, which indicated that single inverse-induction was not an effective method to enrich TICs, at least in A549. We next asked whether inverse-induction combined with drug treatment might enrich TICs from A549. Paclitaxel, a common clinical chemo-

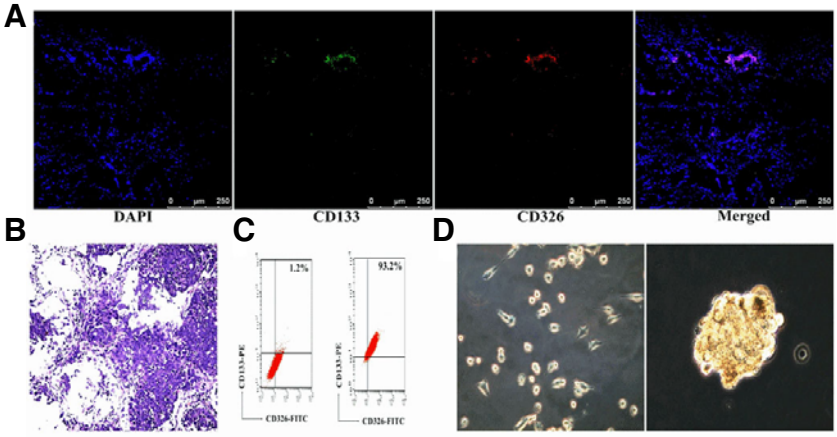

Fig. 3. $CD133^+/CD326^+$ subpopulation co-located in primary tumor samples and sorted by MACS. (A) Immunofluorescence of CD133 (green) and CD326 (red) on fresh sections. (B) Postoperative lung cancer samples were confirmed to be adenocarcinoma by histology. (C) Confirmation of expression by flow cytometry. Positivity for CD133/CD326 of cells before sorting was 1.2% (left) and of cells after sorting was 93.2% (right). (D) Cell morphology: before sorting, cells were adherent (left), and after sorting, cells grew into tight spheres. (right, 200×).

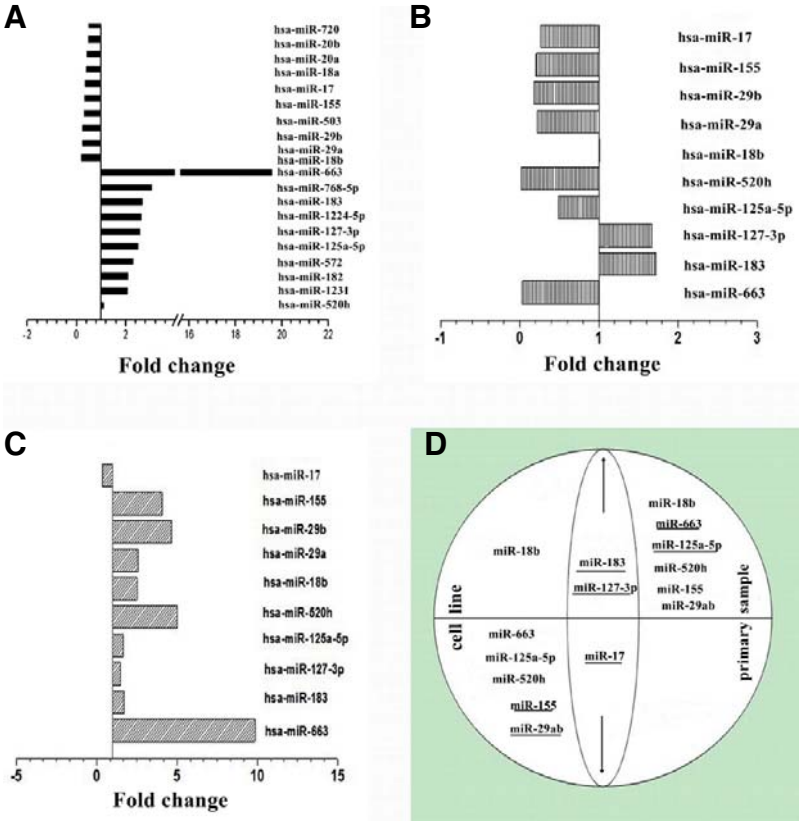

Fig. 4. Aberrant expression of miRNAs in the $CD133^+/CD326^+$ subpopulation derived from A549 cells and validated by qRT-PCR. (A) Partial relative miRNAs exhibited a greater than 2-fold expression change in the $CD133^+/CD326^+$ subpopulation compared to normal A549 cells (defined as 1) by microarray. (B) Validation of expression of 10 selected miRNAs expression in $CD133^+/CD326^+$ cells at the cell line level and (C) further investigation on primary samples by qRT-PCR. (D) Venn analysis shows the up-regulation and down-regulation of the miRNAs in the A549 cell line (left) and primary samples (right). Trends consistent with array data were underlined, and the overlapping area shows validation of miRNA expression in primary samples that showed trends consistent with those in the A549 cell line.

therapeutic used to treat lung cancer, was chosen to be the candidate. Interestingly, after paclitaxel treatment, the surviving cells could gradually regrow into spheres in approximately two weeks. Using flow cytometry, we found that enriched cells highly expressed $CD133^+/CD326^+$, possessed capacities of differentiation, stronger tumorigenesis, higher expression of stem cell-associated genes, and could therefore represent TICs of A549. To confirm whether this subpopulation resides in primary samples, we performed immunofluorescence on fresh tumor samples found that $CD133^+/CD326^+$ cells co-localized in the tumor mass.

Emerging evidence reveals that miRNAs can be important regulators of TICs, and better recognition of aberrant miRNAs in TICs may help explain the potential mechanism of tumorigenesis, therapy-resistance, recurrence and metastasis (Shi et al., 2010; Wang et al., 2010; Wong et al., 2010; Yu et al., 2010). Because little research has been carried out on miRNAs in lung TICs, we performed a miRNA array analysis and qRT-PCR to screen miRNA expression in TICs from A549 cell line compared to the normal cancer cells. We found that miR-29a, miR-29b, miR-17 and miR-155 were down-regulated, and miR-183 and miR-127-3p up-regulated. Among these miRNAs, miR-17, miR-183 and miR-127-3p were further validated in primary specimens. In different tumor types including synovial sarcoma, rhabdomyosarcoma, and colon cancer, miR-183 expression represented a fundamental event in the tumorigenesis and

invasion by targeting EGR1 and PTEN, suggesting that miR-183 has a potential oncogenic role (Saver et al., 2010). In 801D cells (a highly metastatic human pulmonary giant cell line), the opposite result was shown: up-regulation of miR-183 repressed invasion and migration through targeting Ezrin, indicating that miR-183 plays a tumor suppressor role in metastasis (Wang et al., 2008). In pancreatic cancer stem cells, over-expression of miR-183 negatively regulates ZEB1, thereby eliminating some traits of CSCs including the frequency of $CD24^+$ expressing cells, capacity to form spheres and chemoresistance (Wellner et al., 2009). These results were consistent with another study on breast cancer and normal stem cells (Shimono et al., 2009). Several studies reported that miR17-92 cluster, including miR17-5p, miR17-3P, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1, played an oncogenic role in tumor progression. In HEK3293T cells, miR-17-5p was a key regulator of the G1/S phase cell cycle transition and over-expression of miR-17-5p promoted the proliferation of cells by altering MAPK signal transduction (Cloonan et al., 2008). In contrast with the above study, over-expression of miR-17-5p repressed cellular proliferation, adhesion and migration by targeting fibronectin (Shan et al., 2009). By targeting A1B1, miR-17-5p decreased estrogen receptor-mediated and independent gene expression and repressed proliferation of breast cancer cells, suggesting that it has a role as a tumor suppressor in breast cancer cells (Hossain et al., 2006). More recently, it was reported that miR-17-5p could potentially be used to modify the immune tolerance through binding STAT3 in tumor-associated myeloid-derived suppressor cells and might be a potential clinical immunotherapy target (Zhang et al., 2011). In this study, though bioinformatic analysis, we showed that the target genes of these miRNAs are involved in regulating cell cycle, signal transduction, proliferation, apoptosis and migration. For example, miR-183, up-regulation in $CD133^+/CD326^+$ subpopulation might repress RALA, PLAG1 and FOXP1 expression to function in anti-tumorigenesis, but also might repress EGR1 and BTG1 expression to function in pro-tumorigenesis. Similar to miR-183, miR-17 down-regulation in this subpopulation might enhance the expression of oncogenes such as AML1, CCND1, ETS1, CRK, USP6, RAB and PLAG1, and result in pro-tumorigenesis while also enhancing the expression of tumor suppressors such as TP53INP1 and A1B1, resulting in anti-tumorigenesis. Thus, the exact roles of these miRNAs in regulating the bio-behavior of lung tumor-initiating cells remain to be elucidated through future functional validation.

In summary, we successfully separated and identified a distinct cellular subpopulation marked by $CD133^+/CD326^+$ that could represent TICs of the A549 cell line. Next, we identified aberrant miRNA expression in this subpopulation by microarray and qRT-PCR. In future research, the functions of these aberrant miRNAs must be confirmed in TICs. Using our data, we hope to increase knowledge of the relationship between miRNAs and TICs in lung adenocarcinoma.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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