# Identification of CD90 as a marker for lung cancer stem cells in A549 and H446 cell lines

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Received July 17, 2013; Accepted August 30, 2013

## DOI: 10.3892/or.2013.2784

Abstract. Accumulating evidence supports that cancer stem cells (CSCs) are responsible for tumor initiation, progression, distal metastasis and even drug resistance. Although CD90 has been identified as a marker for several types of stem cells, such as liver CSCs, the potential role of CD90 as a marker for lung CSCs has yet to be fully characterized. Our previous study demonstrated that the lung cancer stem-like cells isolated from A549 tumor spheres, which were cultured in serum-free conditioned medium, had stronger proliferation and self-renewal abilities, and expressed higher levels of the stem cell markers Sox2 and Oct4 as compared to A549 adherent cells. In the present study, we identified CD90 as a novel surface marker of CSCs in lung cancer cells. Furthermore, we isolated CD90<sup>+</sup> CSCs from lung cancer cell lines A549 and H446. Our results revealed that the CD90<sup>+</sup> cells, but not the CD90<sup>-</sup> cells, from lung cancer cells displayed higher tumorigenic capacity. These findings suggest that CD90 could be a potential marker of lung CSCs and thus provide new insight into further therapeutic strategies of lung cancer.

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*Abbreviations:* CSCs, cancer stem cells; NSCLC, non-small cell lung cancer; FGF, fibroblast growth factor; EGF, epidermal growth factor; SEM, scanning electron microscopy; FACS, fluorescence-activated cell sorting assay; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

Key words: CD90, cancer stem cell, lung cancer, tumor sphere

## Introduction

Lung cancer, of which 80-85% of cases are non-small cell lung cancer (NSCLC), is at present the leading cause of cancerrelated mortality in both men and women worldwide (1). Adequate therapeutic regimens are largely dependent on the early diagnosis and surgery remains the preferred treatment for early-stage patients (2). However, it has been reported that ~70% patients have advanced local invasion and/or distant metastasis when they are diagnosed and lose the opportunity for surgery. Thus, cisplatin-based doublet chemotherapy has commonly been recommended as the standard regimen for these advanced patients (3). However, the 5-year relative survival rate in the past 30 years has remained at 11-17% for these lung cancer patients (4), in which the low efficacy of chemotherapy (20-30%) is the major cause. Hence, understanding the mechanism underlying chemotherapy and radiotherapy failure is of great importance.

Tumor stem cell theory has provided us new insight into developing strategies for the treatment of malignancies. Emerging evidence indicates that cancer stem cells (CSCs) contribute to tumor initiation, maintenance, metastasis and drug resistance (5-8). To date, CSCs have been identified in several types of malignancies including leukemia (9), brain tumor (10), breast (11) and prostate cancer (12,13-15). CSCs have been characterized in lung cancer by using a variety of stem cell markers (13,16-18), including CD133 (16). However, a recent study demonstrated that both CD133<sup>+</sup> and CD133<sup>-</sup> cells contain CSCs in the lung cancer cell lines A549 and H446 (19), indicating that new markers with higher specificity and accuracy in identifying lung CSCs should be further explored.

CD90 (Thy-1) is a 25-37 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein expressed mainly in leukocytes and is involved in cell-cell and cell-matrix interactions (20). CD90 expression was identified in murine breast CSCs (21), primary high-grade glioma CSCs (22), and in liver malignancy (23). Our previous study (24) initially demonstrated that the A549 tumor sphere cells had a stronger capacity for proliferation and self-renewal, and expressed higher levels of stem cell markers Sox2 and Oct4 than A549 adherent cells. In the present study, we carried out flow cytometry analysis and other experiments to explore whether CD90 could be a marker for identification of lung CSCs.

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# Materials and methods

Cell and tumor sphere culture. Human lung cancer cell lines A549 and H446 (Shanghai Institute of Cell Biology, China) were used in this study. The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco).The tumor spheres were cultured in serum-free conditioned medium that contained DMEM/F12 medium (Gibco), 20  $\mu$ l/ml B27 supplement (Gibco), 20 ng/ml basic fibroblast growth factor (FGF) and 20 ng/ml epidermal growth factor (EGF) (both from PeproTech, USA). All cells were incubated at 37°C with 5% CO<sub>2</sub> and 100% humidity. The third-generation sphere cells were used for further experiments.

Colony and sphere formation. Colony and sphere formation were used to compare the proliferation capability between the  $CD90^+$  and  $CD90^-$  cells. Briefly, cells were resuspended in singular form from both A549 and H446 cell lines and were seeded in 6-well plates with different density: 200 cells/well in A549 cells and 1,000 cells/well in H446 cells, respectively. Two microliters RPMI-1640 culture medium containing 10% FBS were added to each well, and the cells were incubated for 2 weeks at 37°C with 5% CO<sub>2</sub> and 100% humidity. Then, the medium was aspirated off, the wells were washed 3 times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 min. After staining with Giemsa for 5 min, the number of colonies in each group was calculated to compare the colony formation ability (each colony should contain at least 50 cells).

The method of sphere formation was described above. CD90<sup>+</sup> and CD90<sup>-</sup> cells were firstly sorted by fluorescenceactivated cell sorting assay (FACS) and then cultured in serum-free medium. One week later, the number of spheres (each sphere containing at least 50 cells) in each group was calculated under an inverted microscope (BX-40; Olympus, Japan).

*FACS analysis*. The cells and tumor spheres were dissociated as single cell suspension, and washed by PBS and then labeled with antibodies, including CD133 antibody (Miltenyi Biotec, Germany), mouse anti-human CD90 (BD Biosciences, San Jose, CA, USA). The cells were labeled with these antibodies at  $10 \,\mu$ l/1x10<sup>6</sup> cells at 4°C in the dark for 30 min followed by washing with PBS, and then the samples were acquired and analyzed by flow cytometry (FACSAria II; BD Biosciences).

Scanning electron microscopy (SEM). The cells or spheres were fixed using 2.5% glutaraldehyde for 20 min and washed with PBS. Then, the samples were immerged in ethanol to dehydrate and then *tert*-butyl alcohol to displacement. The samples were mounted on aluminum stubs with adhesive tabs and sputter coated with ~30 nm thickness of gold. The samples were observed under SEM (S-3400N II; Hitachi, Japan).

*Immunofluorescent staining*. Immunofluorescent staining was performed to compare the expression of Sox2 and Oct4 in CD90<sup>+</sup> and CD90<sup>-</sup> cells from both A549 and H446 cell lines. The CD90<sup>+</sup> cells and CD90<sup>-</sup> cells were first isolated by FACS followed by fixation in 4% paraformaldehyde for

10 min. The cells were then washed with PBS and permeabilized with 0.3% Triton X-100/PBS for 15 min at room temperature, and incubated overnight at 4°C with mouse anti-Sox2 (Novus Biologicals, LLC) and rabbit anti-Oct4 (Sigma, USA). Secondary antibodies used were Cy3-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-rabbit IgG (Beyotime, China) for 1 h at room temperature. The cell nuclei were counterstained with Hoechst 33342. The cells were visualized under a laser confocal microscope (SP-5; Leica Microsystems, Germany).

*RT-PCR*. Total RNA was isolated by RNAiso reagent (Takara, Japan). Reverse transcription and RT-PCR (Fermentas, USA) were used to observe the expression of stem genes in all samples.  $\beta$ -actin mRNA expression was taken as an internal control. The stem cell genes, including Oct4 and Sox2, were: Oct4 forward, 5'-GCAGCGACTATGCACAACGA-3' and reverse, 5'-CCAGAGTGGTGACGGAGACA-3'; Sox2 forward, 5'-CATCACCCACAGCAAATGACA-3' and reverse, 5'-GCTCCTACCGTACCACTAGAACTT-3';  $\beta$ -actin forward, 5'-TCAAGATCATTGCTCCTCCTG-3' and reverse, 5'-CTGCTTGCTGATCCACAACTAGAACTT-3';  $\beta$ -actin forward, 5'-CTGCTTGCTGATCCACATCTG-3'. PCR was run for 30 cycles with 15 sec/95°C denaturation, 20 sec/58°C annealing and 20 sec/72°C elongation. A melting curve analysis was performed after amplification to confirm the accuracy of the amplification.

*Xenografts*. To explore the tumorigenicity of A549 adherent cells and A549 sphere cells, theses 2 types of cells were injected into the different sides of 4-week-old nude mice (Laboratory Animal Center, The Third Hospital of Third Military Medical University) at doses of  $1 \times 10^6$ ,  $1 \times 10^5$ ,  $1 \times 10^4$  and  $5 \times 10^3$  cells. After 8 weeks, the tumors were removed and measured. The same method was applied to compare the tumorigenicity of CD90<sup>+</sup> and CD90<sup>-</sup> cells from A549 cell lines. However, the doses of CD90<sup>+</sup> cells or CD90<sup>-</sup> cells injected into nude mice were  $5 \times 10^4$ ,  $1 \times 10^4$  and  $5 \times 10^3$  cells.

# Results

SEM reveals different features in A549 adherent and sphere cells. Tumor spheres formed in serum-free medium were considered to be feasible and effective in the enrichment of CSCs (25-27), which had also been used to enrich lung CSCs in our previous study (24).

In the present study, SEM was used to directly observe and compare the surface morphology of both A549 adherent and sphere cells. As shown in Fig. 1, there were several protrusions on the surface of A549 adherent cells which also exhibited high proliferation ability (Fig. 1A and B), while the surface of A549 sphere cells was smooth (Fig. 1C and D), and most of these cells were undifferentiated. Therefore, morphological features between these 2 types of cells suggest that the sphere cells exhibit stem-cell characteristics.

The percentages of CD90 are higher in tumor sphere than adherent cells. Our previous study suggested that sphere cells exhibited stem-cell characteristics, but the markers identifying these lung CSCs needed to be further explored. It is highly controversial whether CD133 is a molecular marker for lung



Figure 1. Scanning electron microscopy (SEM) analysis reveals different features between A549 adherent and sphere cells. (A and B) A549 adherent cells; (C and D) A549 sphere cells. (A and B) Several protrusions on the surface of A549 adherent cells are shown, which also exhibited high proliferation ability, while the surface of A549 sphere cells was smooth. (C and D) These sphere cells tightly linked with each other, most which were undifferentiated. Therefore, morphological features between these 2 types of cells indicated that the sphere cells exhibited stem-cell characteristics.



Figure 2. Flow cytometry analysis of cell surface markers. The percentage of  $CD133^+$  cells and  $CD90^+$  cells were 0.1 and 0.9% in A549 adherent cells, respectively, whereas the percentage of  $CD133^+$  cells and  $CD90^+$  cells were ~5.4 and 41.2% in A549 sphere cells. The fluorescence-activated cell sorting assay (FACS) results demonstrated that both CD133 and CD90 were significantly higher expressed in spheres cells.

CSCs, and CD90 received increasing attention as a new CSC marker in several types of malignancies. Thus, we speculated that CD90 may present a new marker for lung CSCs.

To verify this hypothesis, FACS assay was first performed to detect the expression of CD133 and CD90 in both A549 adherent cells and A549 sphere cells. As shown in Fig. 2,



Figure 3. The colony formation of CD90<sup>+</sup> and CD90<sup>-</sup> cells from the A549 and H446 cell lines. CD90<sup>+</sup> and CD90<sup>-</sup> cells were isolated from A549 and H446 cell lines with fluorescence-activated cell sorting (FACS) assay, respectively. The cells were seeded in 6-well plates and cultured for 2 weeks at  $37^{\circ}$ C, 5% CO<sub>2</sub>. After staining with crystal violet, we calculated the number of colonies formed. The results showed that in both A549 or H446 cells, the number of clones formed by CD90<sup>+</sup> cells were more than those formed by CD90<sup>-</sup> cells ( $^{\circ}$ P<0.05).



Figure 4. The sphere formation of  $CD90^+$  and  $CD90^-$  cells from A549 and H446 cell lines. The results showed that the spheres formed by  $CD90^+$  cells were superior in number, volume and morphology than those formed by  $CD90^-$  cells in either the A549 or the H446 cell line.

the fractions of CD133<sup>+</sup> cells in tumor spheres and adherent cells were 5.4 and 0.1%, respectively (Fig. 2A and B). Correspondingly, we also found that the percentages of CD90<sup>+</sup>

cells were 41.2 and 0.9% in tumor spheres and adherent cells, respectively (Fig. 2C and D). As CD90 was highly expressed in A549 sphere cells, but low in A549 adherent cells, a percentage



Figure 5. The expression of Oct4 and Sox2 in CD90<sup>+</sup> and CD90<sup>-</sup> cells. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed to test the expression of stem cell-related genes Oct4 and Sox2. The results revealed that both Oct4 and Sox2 were significantly higher expressed in CD90<sup>+</sup> cells than in CD90<sup>-</sup> cells from either the A549 or the H446 cell line (P<0.05).

considerably higher than CD133, CD90 may present a potential marker for A549 sphere cells, rich in lung CSCs.

The colony and sphere formation of  $CD90^+$  and  $CD90^-$  cells from A549 and H446 cell lines. We previously tested the proliferation ability between A549 adherent cells and A549 sphere cells using colony formation and MTT assay, by which we showed the A549 sphere cells have a stronger proliferation ability (P<0.05) (24). In the present study, the colony formation assay was used to detect the proliferation ability of both  $CD90^+$  and  $CD90^-$  cells isolated from both A549 and H446 cell lines. The number of  $CD90^+$  cell colonies was higher and the size was larger than  $CD90^-$  cells both in the A549 and the H446 cell line (P<0.05) (Fig. 3).

To further determine whether the CD90<sup>+</sup> cells had stronger sphere formation capacity, both CD90<sup>+</sup> and CD90<sup>-</sup> cells were firstly sorted from A549 and H446 cell lines with FACS assay and then cultured in the serum-free conditioned medium. The results showed that the number and volume of spheres formed by CD90<sup>+</sup> cells were superior to those formed by CD90<sup>-</sup> cells in both A549 and H446 cell lines. It is worth mentioning that the cells in the spheres formed by CD90<sup>+</sup> cells clustered more closely with regular shape than those in the spheres formed by CD90<sup>-</sup> cells (Fig. 4). Thus, the cell spheres formed by CD90<sup>-</sup> cells are inferior to those formed by CD90<sup>+</sup> cells both in quality and quantity, indicating that CD90<sup>+</sup> cells displayed stem-cell characteristics.

 $CD90^+$  cells express higher levels of stem cell genes Oct4 and Sox2. The embryonic markers, Sox2 and Oct4, play an important role in the growth and metastasis of lung cancer (28,29), and are candidate stem cell-related genes to study lung CSCs. Our previous study demonstrated that both Sox2 and Oct4 are highly expressed in A549 sphere cells than A549 adherent cells (24). In the present study, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was employed to analyze stem-cell related gene expression profiles of CD90<sup>+</sup> and CD90<sup>-</sup> cells isolated from both A549 and H446 cell lines. Results showed that the mRNA expressions of either Oct4 and Sox2 in CD90<sup>+</sup> cells were markedly higher than those in CD90<sup>-</sup> cells (P<0.05) (Fig. 5). Immunofluorescent staining was also performed to further confirm the protein levels of Oct4 in both CD90<sup>+</sup> and CD90<sup>-</sup> cells sorted from A549 or H446 cell lines. The results demonstrated that the Oct4 protein level was markedly higher in CD90<sup>+</sup> cells (Fig. 6). Taken together, we concluded that CD90<sup>+</sup> cells, but not CD90<sup>-</sup> cells, are characterized as stemlike cells.

Xenograft experiments reveal CD90<sup>+</sup> cells have a higher tumorigenicity than CD90<sup>-</sup> cells. The effect of CD90<sup>+</sup> and CD90<sup>-</sup> cells on tumor formation was further investigated in vivo. In the present study, tumorigenicity was defined as the capacity of the cells with certain number, following serial dilution, to form tumor nodules in immunodeficient mice within a certain time interval (8 weeks). We first established the xenograft model to assess the tumorigenic capacity of A549 sphere cells as compared to A549 adherent cells as control. Nude mice were divided into 4 groups randomly and were subcutaneously inoculated under bilateral axillaries with different doses of cell numbers as described in Materials and methods. Eight weeks later, tumor nodules appeared in one nude mouse from 5,000 inoculated A549 sphere cells, but there were no nodules found in the control side. When the cell number was increased to 1x10<sup>5</sup>, all 5 nude mice generated tumor nodules formed by A549 sphere cells, while the remaining 2 out of 5 nude mice generated tumor nodules in the control (Table I). In addition, the tumor nodules induced by A549 sphere cells were considerably bigger than those formed by A549 adherent cells (Fig. 7A and B).

Using the same method, we further compared the tumorigenic capacity between CD90<sup>+</sup> and CD90<sup>-</sup> cells both isolated from A549 cell lines. Tumor nodules from CD90<sup>+</sup> cells appeared after 8 weeks from  $5x10^3$  inoculated cells. When the cell number was increased to  $1x10^4$ , 4 out of 5 nude mice generated tumor nodules. By contrast, the CD90<sup>-</sup> cells did not induce tumor formation in nude mice, even after injecting  $1x10^4$  cells (Table II). Furthermore, the tumor nodules induced by CD90<sup>+</sup> cells were much bigger than those formed by CD90<sup>-</sup> cells (Fig. 7C). These results strongly indicate that A549 sphere cells have a higher tumorigenic capacity, and CD90<sup>+</sup> cells but not CD90<sup>-</sup> cells are stem-like cells.



Figure 6. Immunofluorescent staining shows Oct4 expression in CD90<sup>+</sup> and CD90<sup>-</sup> cells. Immunofluorescent staining was used to further confirm the Oct4 expression in CD90<sup>+</sup> and CD90<sup>+</sup> and CD90<sup>+</sup> and H446 cell lines. Laser confocal microscopy showed the expression of Oct4 was higher in CD90<sup>+</sup> cells (Oct4-positive expression located in the nucleus, dyed red).



Figure 7. Tumorigenic capacity of the A549 or A549 sphere cells. (A and B) We showed the nodules formed by A549 sphere cells were considerably bigger than those in the control group while the same numbers of cells  $(1x10^5)$  were injected. Tumorigenic capacity of the CD90<sup>+</sup> and CD90<sup>-</sup> cells from A549 sphere cells is shown in (C). Tumor nodules induced by CD90<sup>+</sup> cells (left) were much bigger than those formed by CD90<sup>-</sup> cells (right). Both sides were injected subcutaneously with  $5x10^4$  cells. Arrows indicate the site of cell injection and the tumor nodules.

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	Cell no.			
Cell type	1x10 <sup>6</sup>	1x10 <sup>5</sup>	1x10 <sup>4</sup>	5x10 <sup>3</sup>
A549	5/5	2/5	0/5	0/5
A549 sphere	5/5	5/5	2/5	1/5

Table I. Comparison of tumorigenic capacity of the A549 adherent and sphere cells.

Table II. Comparison of tumorigenic capacity of the CD90<sup>+</sup> and CD90<sup>-</sup> cells from A549 cell lines.

Cell type		Cell no.	
	5x10 <sup>4</sup>	1x10 <sup>4</sup>	5x10 <sup>3</sup>
A549 CD90 <sup>-</sup>	3/5	0/5	0/5
A549 CD90+	5/5	4/5	1/5

A549 adherent and sphere cells were injected subcutaneously into bilateral axillaries of the same nude mouse, respectively. After 8 weeks, all 5 nude mice injected with A549 sphere cells (cell number,  $1x10^5$ ) formed tumor nodules, while 2 out of 5 mice formed tumor nodules in the control.

The CD90<sup>+</sup> and CD90<sup>-</sup> cells were isolated from the A549 cell line, and each cell type was injected subcutaneously into 2 different sites of the same nude mouse. Eight weeks later, the CD90<sup>+</sup> cells (cell number,  $5x10^3$  even  $1x10^4$ ), but not the CD90<sup>-</sup> cells, formed tumor nodules. When the number of cells was increased to  $5x10^4$ , all 5 nude mice injected with CD90<sup>+</sup> cells induced tumor nodules, while 3 out of 5 nude mice injected with CD90<sup>-</sup> cells formed tumor nodules.

#### Discussion

In our previous study, tumor spheres successfully cultured in the serum-free medium were used to enrich and identify the lung cancer stem cells (CSCs) in the A549 cell line. The results showed that, compared with their adherent cells, tumor sphere cells had higher expression of stem genes Oct4 and Sox2. Therefore, we concluded that CSCs could be enriched in tumor sphere and such an enrichment process was an effective and convenient method for screening and identifying tumor stem cells. In the present study, we identified CD90 as a novel unique surface marker for further isolation and identification of CSCs from lung cancer cell lines A549 and H446. Our results revealed that CD90<sup>+</sup> cells, but not CD90<sup>-</sup> cells, from lung cancer cell lines highly expressed stem cell markers, Oct4 and Sox2, and displayed higher proliferative and tumorigenic capacity. Therefore, CD90 is a promising new marker for lung CSCs.

The CSC theory has been widely recognized due to the continual emergence of new evidence. At present, the origin of the CSCs remains unclear, but emerging evidence suggests that CSCs play an important role in the occurrence and development of malignant tumors. Tumor stem cells have also been considered as the major cause of drug resistance to chemotherapy and radiotherapy failure (7,30-33). Surface molecular markers can be used for flow cytometry screening of a unique subset in a heterogeneous population of cells such as cancer cells. CD133 is expressed in a variety of tumors as a recognized marker for tumor stem cells such as brain tumor (10,34), breast cancer (11) prostate cancer (12), colon cancer (14), pancreatic cancer (35), hepatocellular (36) and renal carcinoma (37). However, CD133 remains highly controversial as a tumor stem cell marker (38-40). A previous study showed that both CD133<sup>+</sup> and CD133<sup>-</sup> subpopulations of A549 and H446 cells contain cancer-initiating cells (19), which indicated that CD133 may not be an ideal marker for lung CSCs. Therefore, as the identification of lung CSCs is fundamental and vital for further research, for example, the mechanism of lung CSCs involved in the drug resistance to chemotherapy and radiotherapy, more specific and accurate markers for identifying lung CSCs are urgently required.

CD90 has been characterized as a surface molecular marker of CSCs in various malignancies including glioblas-

toma and liver cancer (22,23). In our previous experiments, we found the proportion of CD90<sup>+</sup> in lung cancer tumor sphere cells higher than in the adherent cells. Therefore, we isolated the CD90<sup>+</sup> and CD90<sup>-</sup> cells from the lung cancer cell lines A549 and H446. We found that the numbers of the clones and spheres formed by CD90<sup>+</sup> cells were more than those formed by CD90<sup>-</sup> cells (P<0.05). The expressions of stem cell-related genes Oct4 and Sox2 in CD90<sup>+</sup> were markedly higher than those in CD90<sup>-</sup> cells (P<0.05). Moreover, the animal experiments also indicated that the A549 sphere cells had a higher tumorigenic capacity, and CD90<sup>+</sup> cells were more likely to be stem cells than CD90<sup>-</sup> cells. Therefore, both *in vitro* and *in vivo* experiments indicate that CD90<sup>+</sup> cells have higher proliferation, self-renewal and tumorigenic capacity, and CD90<sup>+</sup> is a novel marker of lung CSCs.

In conclusion, we demonstrated that serum-free conditioned culture can be effectively employed for enrichment of CSCs, and tumor spheres are suitable for studying CSCs. Meanwhile, our preliminary results revealed the CD90<sup>+</sup> cells, but not CD90<sup>-</sup> cells, can be characterized with stem-like features. The expression of CD90<sup>+</sup> in human lung cancer tissues and primary cells from lung cancer patient specimens require further investigation in future studies. Finally, the identification of CSCs with CD90 will provide a cellular basis to investigate the mechanism of tumorigenesis, drug resistance to chemotherapy and, thus, favor the design of future therapeutic strategies for lung cancer.

### Acknowledgements

We thank Professor Xia Zhang, the PI of Institute of Pathology and Southwest Cancer Center, for the valuable suggestions during the manuscript writing. This study was supported by grants from the National Basic Research Program of China (973 Program, no. 2010CB529402).

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