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Characteristics and Multilineage Differentiation of a Novel Type of Pulmonary Mesenchymal Stem Cells Derived from Goose Embryos

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HIGHLIGHTS

- PMSCs were successfully isolated from the goose embryos lung tissues for the first time.
- PMSCs possessed hereditary stability, self-renewal ability and consistently expressed MSCs associated phenotypes.
- PMSCs possessed cross-embryonic layer differentiation potential.

Abstract: Pulmonary mesenchymal stem cells (PMSCs) are considered important in therapeutic and regenerative responses to lung injury. Despite extensive studies on the human, porcine, sheep, mouse and rat, it was found that PMSCs, which can been performed on goose, have not been well explored. Elucidation of cell differentiation, proliferation, which have not yet been well described, may promote the development of injured lung therapy. In our study, we firstly disclosed biological characteristics of spindle-shaped PMSCs from pulmonary tissues of goose embryos. Growth kinetics and counting kit-8 (CCK8) assay were employed for proliferative activity evaluation. When appropriately induced, PMSCs could differentiate into osteoblasts, adipocytes, chondrocytes, type II alveolar epithelial cells (ATII), which demonstrated that PMSCs have cross-embryonic layer differentiation potential. Besides, undifferentiated PMSCs consistently expressed MSCs

associated phenotypes, such as CD166, CD90, CD44, CD29, CD71 and CD105, as identified by immunohistochemistry and RT-PCR. Karyotype analysis demonstrated that PMSCs possessed stable diploid karyotype. Collectively, we successfully established applicable methods for isolation, culture, identification and characterization of goose PMSCs which suggested a potential therapeutic use in regenerative therapy.

Keywords: PMSCs; Proliferation; Cell differentiation; Biological characterization; Regenerative therapy.

INTRODUCTION

Tissue specific mesenchymal stem cells (MSCs) isolated from a host of recognized sources is considered to be a promising alternative in cell-based therapy because they are capable of release of paracrine factors to reduce the level of inflammation, inhibit the growth of bacterial and enhance repair of injured tissue and because they may self-renewal and differentiate into various cell lineages [1-3]. In addition to practical advantage mentioned above, MSCs had a tendency to migrate into inflamed or injured sites to repair diseases associated with autoimmune disorders, inflammation or regenerative medicine [4]. Crippling and devastating chronic pulmonary diseases are epidemic world-wide and constitute the major causes of human death. In particular, with prevalence rising, chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis can lead to the acceleration of lung ageing and rank third in a survey on cause of chronic-disease mortality [5, 6]. Based on extensive clinical trials, therapeutic potential of MSCs hold promise for ameliorating the function of damaged lung. In the lung, reparative effects investigation of extra-pulmonary MSCs has originally drawn the attention of researchers, such as the most well-studied BM-MSCs or adipose-derived MSCs which can reduce injured lung to some extent [7-10].

The therapeutic responses of reliable MSCs derived from specific pulmonary tissues in lung injury models treatment has also been revealed over time. In investigation on mice, pulmonary-derived MSCs were also verified to ameliorate lung fibrosis caused by bleomycin or deliver paracrine signals to protect against elastase-injured lung tissues [11, 12]. Currently, more studies have observed greater survival, higher lung retention, poorer phagocytosis and more interactions with endothelial cells of transplanted PMSCs to elastase-injured lung compared to BMSCs-treated mice. In addition to these significant difference, tissue specific PMSCs expressed more engraftment-related functional surface proteins (ICAM-1, PDGFRα, and Itg α2) that modulate adherence, invasion and migration [13]. Further, Khatri and coauthors also documented that Lung-derived MSCs may be potentially more effective for pulmonary diseases therapies in comparison to BM-MSCs [14].

Many investigations have disclosed that pulmonary-derived mesenchymal stem cells has been elucidated on the human, sheep, porcine, mouse and rat; however, PMSCs from goose has little been discussed. In our study, we firstly identified and characterized MSCs from the pulmonary tissues of goose. Herein, PMSCs of goose demonstrated are in accordance with PMSCs from human in terms of in vitro surface phenotypes and differentiation potential. Further elucidation of biological properties and differentiation potential of PMSCs, which has not been thoroughly explored, may be served as a basis for PMSCs more effective intervention of lung.

MATERIAL AND METHODS

Cell lines

Experimental animal procedures, used in our study, were under approval of Care of Experimental Animals Committee of the Bengbu Medical College. Goose embryos (12 individual) between 18 to 19 days of age were randomly dissected under aseptic conditions. The lungs were fractionated, stringently flushed with phosphate-buffered saline (PBS) for 6 to 8 times, and mechanically minced into small pieces. Collagenase treatment was then carried out for 30 min at 37°C to acquire single-cell suspension. Following filtration with cell strainer, cell pellet were acquired by centrifugation and plated to 24-well culture plates in 2 ml of general culture media (DMEM/F-12, 10% FBS, and 10 ng/ml bFGF) for culture. After 24 h of outgrowth, the supernate of cells containing blood cells and other non-adherent cells were discarded, followed by two washes with PBS. General culture media was refreshed every other day. When primary PMSCs confluency reached 80% to 90%, 0.125% trypsin treatment was processed to passage attached PMSCs at 1:2 dilution.

Cell proliferation, and karyotype analysis

After P7, P23 and P35 PMSCs (1×10^4 cells/well) were seeded in 24-well culture plates, cells were expanded with general culture media incubation for 7 days, cells average number of every three wells were then count and repeated 3 additional times in fields everyday under a microscope. At the end of cells culture,

population doubling times (PDTs) of each passage were calculated based on the formula PDT = (t - t0) lg2/(lgNt – lgN0) (t: termination time; t0: starting time; Nt: ultimate cell number; N0: initial cell number), growth kinetics and CCK8 (Dojindo, Japan) assay were acquired for proliferative activity evaluation. Briefly, in karyotype test, pretreatment of cells harvested included hypotonic treatment with 0.075 M KCL, followed by fixation with methanol/glacial acetic acid (3:1) and Giemsa staining. Chromosome metaphases were further prepared for viewing in 50 to 100 spreads.

Immunofluorescence staining

The third passage of PMSCs grown on cover slips were fixed using 4% paraformaldehyde (PFA). Then, the right amount of volume of 0.25% Triton X-100 was added, which was subsequently blocked with 0.5 h of 10% normal goat serum incubation at RT and incubated at 4℃ with primary antibodies, cell surface markers：CD44, CD29, CD105, CD71 (1:100, Bioss); the type II alveolar epithelial markers： SPC (1:200, Bioss). On the following day, specific binding sites were detected under fluorescent microscope after incubation with Alexa Fluor 488-labeled secondary antibody (1:500, Invitrogen, CA, USA) and DAPI, respectively.

Correspondingly, flow cytometry was performed by the analysis of the extent of cell labeling, followed by overnight incubations with primary antibodies and 1 h incubations with secondary antibody at RT, respectively.

RT-PCR analysis

The levels of cell surface markers mRNA in PMSCs and differentiated cells were detected via RT-PCR. In short, specimens pretreatment included total RNA extraction using TRIzol reagent, followed by cDNA synthesis using a kit (TaKaRa, Japan) and PCR amplification using LA Taq (TaKaRa, Japan). The sequences for all primers in present study were designed in Table 1.

Table 1. Primer sequences used for RT-PCR.

Gene	Primer sequences (5'-3')
CD29	F: AGTGGCCCTGACATCATTCC
	R: ATCACTGCAGTTTGCCCTAA
CD44	F: ATGGGGATGCCAGCAAATCA
	R: AATTCTGGCCCAAGTTCCGT
CD71	F: AAGTCGAGTCTCTTGCTGGC
	R: AGGGAGTCCTAAGCCCAGAG
CD90	F: TCAGCGGAAAGATGAACCCC
	R: CATGTAGACCCCCTCATCGC
CD105	F: TCATCATTGCCAGGTCCCAC
	R: GCACGAAAGCCTCCTTGTTG
CD166	F: GATGCAGTGGGCATGACTCT
	R: AACGCGGAGCAGTGAAAAAC
CD34	F: TCCACCTCGCAGTCTTGAAC
	R: CCGGTTCCGCTAAGTTCTGT
CD45	F: TTCTCACCTCCAAGCACCAC
	R: TGCTAGTGGGGATGGTCTCA
PPAR-γ	F: ACAAGCGGAGAAGGAGAAGC
	R: AGCGGTGACACATGCTTACA
LPL	F: GGCACCTCACGTCTTGGTAA
	R: GGCAATTTGTTGCGGGGATT
COL1A2	F: AAGTTGGCTACGACGCAGAA
	R: GCTGGGTGGCCATATCCTTT
ATF4	F: TCGACGAAAAGACCCTCAGC
	R: AGCCAATTGGAGGAGACTGC
GAPDH	F: GTCAAGGCAGAGAACGGGAA
	R: GGTTCACGCCCATCACAAAC
ACAN	F: GAGATCAGCGGACTTCCGAG
	R: AGTTCACCAGATGCAGAGGC
VIM	F: GCGTCAGGCCAAACAAGAAG
	R: TACTCACGAAGATGGCGAGC
SPC	F: GCACCTCAAACGCCTTCTCA
	R: CTTATAGGCCGTCAGGAGCC

Adipogenic, osteogenic and chondrogenic differentiation of PMSCs

To evaluate the multipotent differentiation potential along the adipogenic lineage, the adipogenic culture media were added with 10% FBS-DMEM/F12 media supplemented with 1.0 M dexamethasone, 200 mM indomethacin, 0.5 mM isobutyl-methylxanthine and 10 mM insulin. Cells were harvested for Oil-Red-O staining for 30 min at room temperature and RT-PCR analysis were acquired for further measurement of the fat droplets and expression of markers, respectively. The cells were cultured in osteogenic media, which consisted of DMEM/F12 and supplements, including 10 mM β-glycerophosphate, 0.5 μM dexamethasone and 50 μM ascorbic acid, for 10 days. Osteogenic differentiation of PMSCs were determined through RT-PCR assay and Alizarin Red staining assay for 30 min at room temperature, respectively. To investigate the chondrogenic ability in PMSC differentiation, 1% insulin-transferrin selenium, in the presence of 40 μg/ml L-proline, 0.1 μM dexamethasone, 1 mM sodium pyruvate, 50 μg/ml L-ascorbic acid, and 10 ng/ml transforming growth factor-β3 were added to the chondrogenic media. After differentiation, induced cells were harvested for Alcian Blue staining for 30 min at room temperature and RT-PCR analysis.

Type II alveolar epithelial cells differentiation of PMSCs

Differentiation media which can promote the differentiation of PMSCs into ATII cells was adopted using PneumaCultTM-ALI maintenance medium (STEMCELL Technologies), which consisted of PneumaCultTM-ALI complete base medium and supplements, including 0.2% Heparin Sodium Salt, Hydrocotisome stock slotion (200×), and maintenance supplement (100 ×). After 10 days of induction in differentiation conditions, differentiation of PMSCs were detected by observing the morphological change and immunofluorescence staining.

Statistical analysis

All experimental data are shown as the means ± standard deviation (SD), and the Student's t-test was used to determine differences between groups. GraphPad Prism 7.0 software was used for statistical analyses.

RESULTS

Expansion of PMSCs

PMSCs were successfully isolated from the lungs of all 6 goose embryos. After 8 days of general culture, plastic-adherent goose PMSCs were observed under an optical microscope, to examine whether PMSCs maintain characteristic features of fibroblast-like morphology of MSCs. Additionally, we found fibroblast-like characteristics of PMSCs were relatively stable on subsequent subculture process (Figure 1). Vacuoles were found in PMSCs after P39 suggesting the onset of senescence of cells (Figure 1).

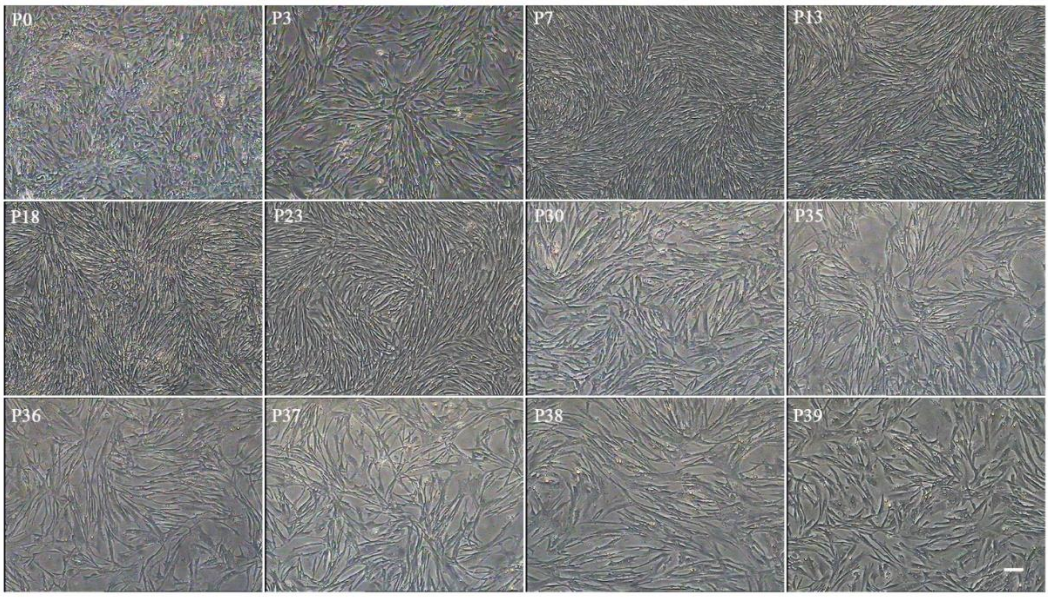

Figure 1. Morphologic images of PMSCs after several passages under general culture conditions. Scale bar = 50 μm.

Karyotype and proliferation assay of PMSCs

To detect the characteristic of diploid cells, karyotype was analyzed via Giemsa staining (Figure 2A). It was found that cultured goose cells maintained its genetic stability and the frequencies from different passages (P7, P23, P35) with 2n = 78 were 94.7%, 92.5% and 91.4%, respectively. PMSCs had high proliferation capacity, and cell growth was typical “S” shapes in vitro as shown in Figure 2B. The PDTs were about 35.5, 38.3 and 42.7 h for P7, P23, P35, respectively. There was no observable difference in the investigation of the cell viabilities of cultured PMSCs at P7, P23 and P35 by CCK-8 assay (P > 0.05, Figure 2C).

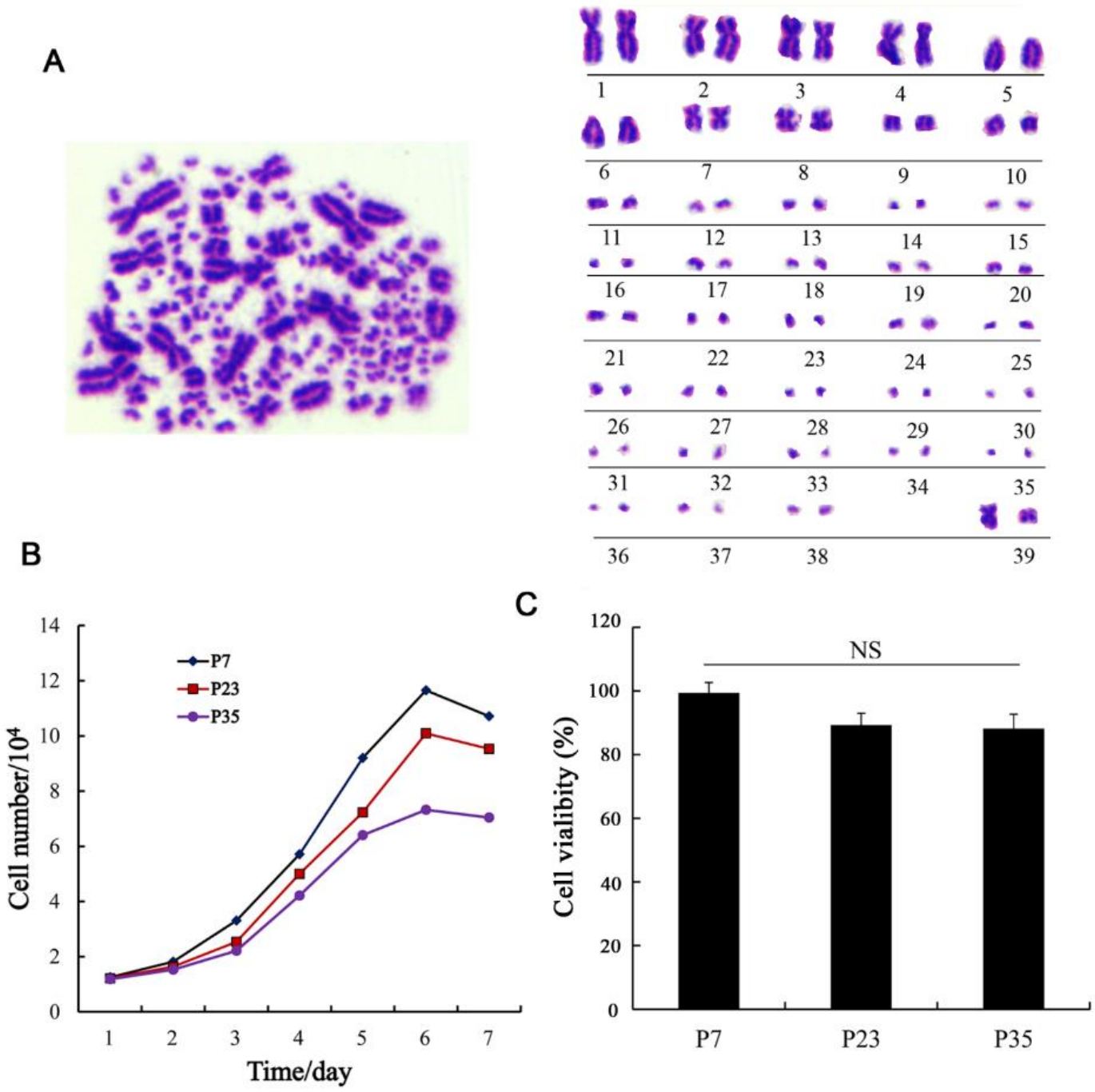

Figure 2. Karyotype and proliferation potential of PMSCs. (A) Karyotype of cultured cells. (B) Growth curves of cultured cells at different passages; (C) Cell viabilities were measured at P7, P23, and P35. NS not significant (P>0.05). **P < 0.01.

Characterization of PMSCs

Many mesenchymal stem cells-related specific phenotypes were investigated, and CD166, CD90, CD44, CD29, CD71 and CD105 were found to be expressed in goose PMSCs by RT-PCR but not hematopoietic markers (CD34, CD45) in normal cultural conditions (Figure 3B). As we confirmed above, immunofluorescence data showed a similar results in expression of CD29, CD44, CD71 and CD105 (Figure 3A). As shown in Figure 3C, the expression of specific markers were similarly observed in PMSCs by flow cytometry and the proportions was found to be achieved over 95%.

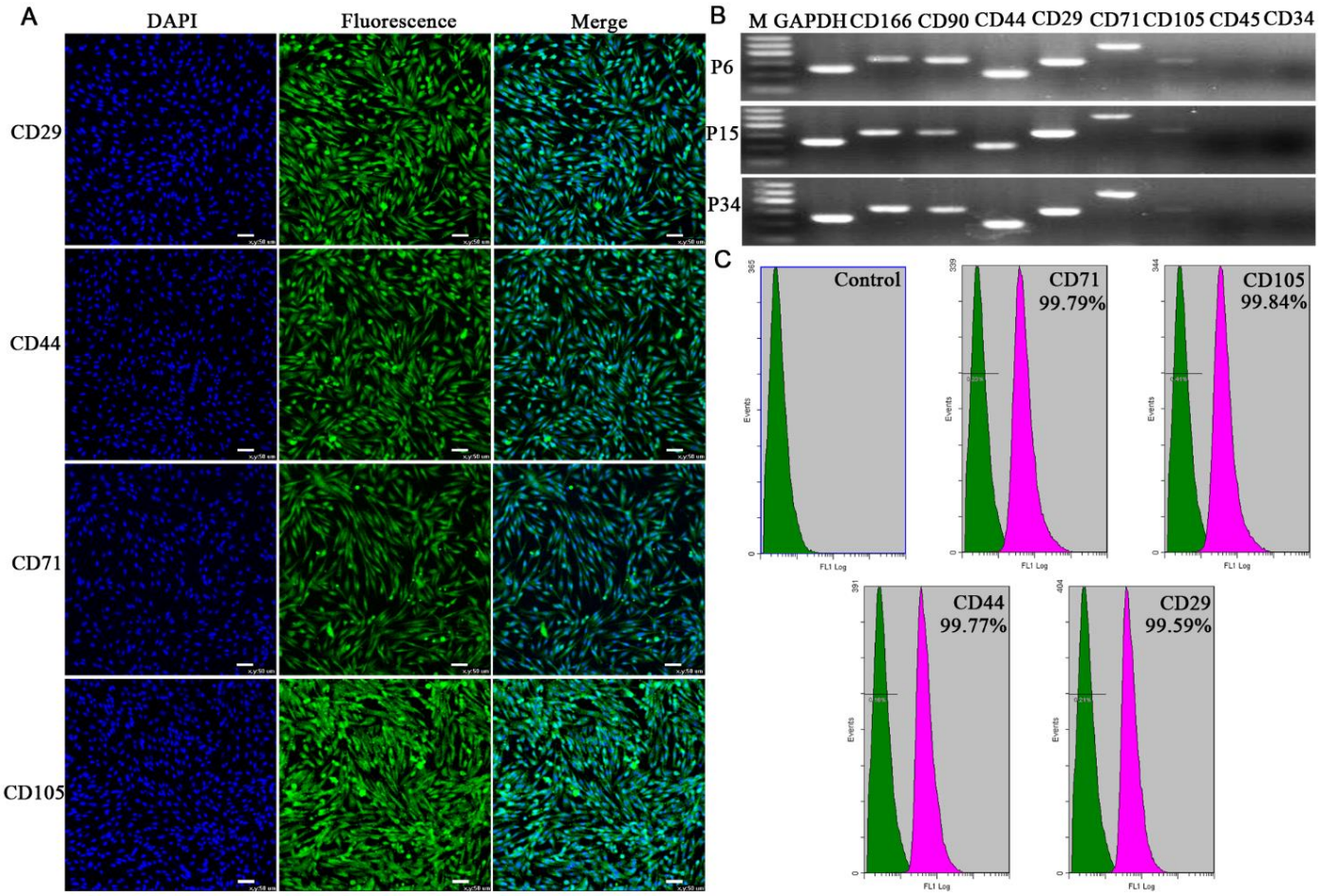

Figure 3. The expression of mesenchymal markers that identify PMSCs were investigated. (A) MSCs-related markers, including CD29, CD44, CD71 and CD105 were expressed in PMSCs by immunofluorescent analysis; (B) Cultured PMSCs were examined for the expression of CD166, CD90, CD44, CD29, CD71 and CD105; (C) PMSCs stained strong with CD29, CD44, CD71 and CD105 markers by flow cytometry.

PMSCs are capable of multiple differentiation

It was found that lipid droplets, which appeared in the cytoplasm of differentiated goose cells, were increased in a time-dependent manner by 3-10 days incubations with differentiation conditions treatment. We then examined the levels of adipogencytes related markers mRNA in differentiated cells and found that PPAR-γ, LPL were positively expressed on the tenth day of differentiation of PMSCs into adipogencytes (Figure 4B). Also, positive Oil-Red-O staining were observed on the tenth day (Figure 4A).

Additionally, we investigated the effect of osteogenic induction medium and general culture media on goose cells through the detection of the expression of COL1A2 and ATF4 in PMSCs by RT-PCR, and COL1A2 and ATF4 was unchanged when PMSCs incubated in normal cultural conditions, but was found to be positively expressed when cultured in differentiation conditions (Figure 4D). Also, positive Alizarin Red staining were observed on the seventh day (Figure 4C).

Under appropriate chondrogenic differentiation treatment, significant differences were observed after 12 days of intervention. We found that positive Alcian Blue staining were observed on the twelfth day (Figure 4E). Also, the expression of VIM and ACAN were verified on the twelfth day, respectively, but PMSCs which were cultured in general culture media remained unchanged (Figure 4F).

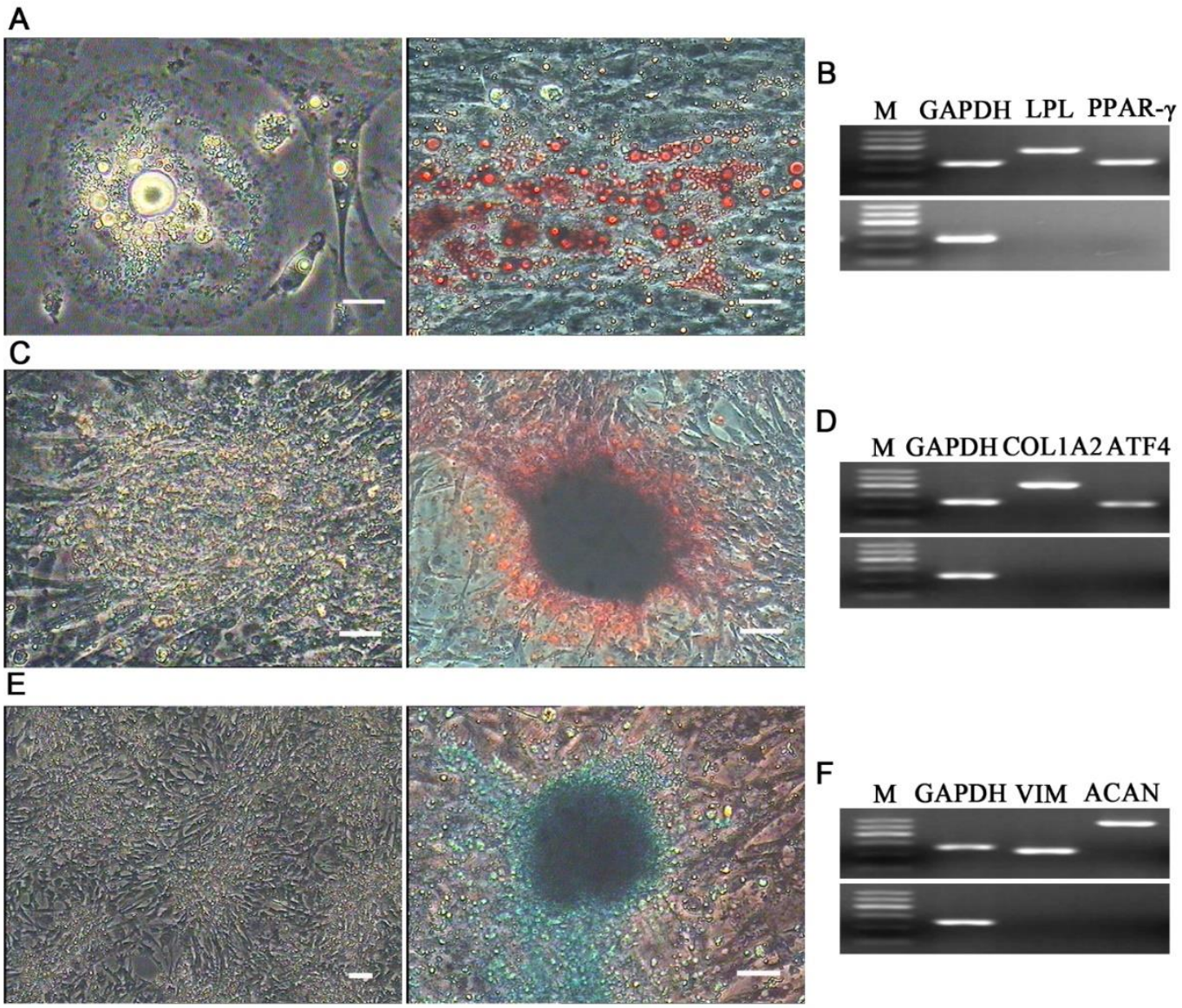

Figure 4. PMSCs differentiate to adipogenic, osteogenic and chondrogenic cells. (A) Lipid droplets within differentiated cells were positive stained after Oil-Red-O treatment; (B) Cells were processed for RT-PCR to analyze PPAR-γ and LPL expression; (C) Calcium within differentiated cells were positive stained after Alizarin Red treatment; (D) Cells were processed for RT-PCR to analyze COL1A2 and ATF4 expression; (E) Chondrogenic differentiation was noticable after Alcian Blue treatment; (F) Cells were processed for RT-PCR to analyze VIM and ACAN expression. Scale bar = 20 μm.

We drove the differentiation of PMSCs into epithelial cells in PneumaCultTM-ALI Maintenance Medium. PMSCs showed morphological changes with epithelial-like cuboidal shape under inverted phase-contrast microscope (Figure 5A). Additionally, induced cells expressed specific markers of type II alveolar epithelial cell including SPC by immunofluorescence staining and RT-PCR assay after 2 weeks differentiation (Figure 5B and 5C). Contrastingly, to results above, we found that goose cells were unchanged in general culture media (Figure 5C).

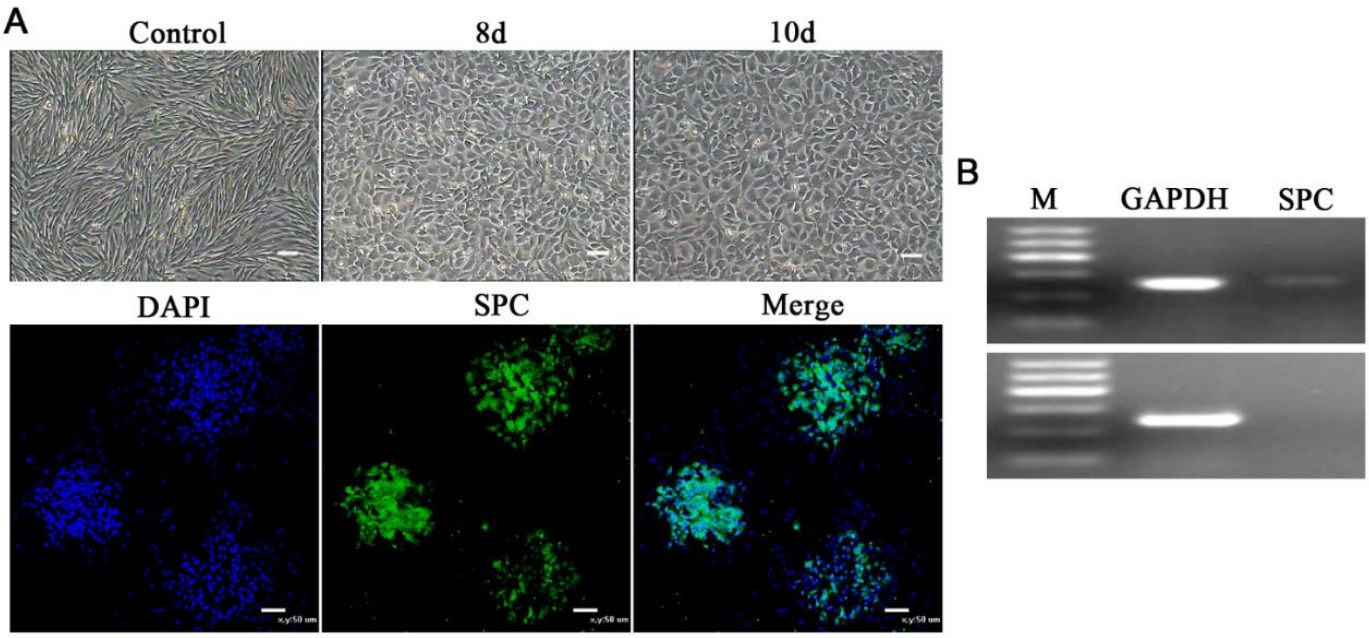

Figure 5. PMSCs differentiate to type II alveolar epithelial cells. (A) Phase-contrast images of PMSCs differentiating into ATII for 0, 8, 10 days. (B) PMSCs, 10 days after induction with differentiation media were immunostained for SPC; (C) RT-PCR detection of SPC expression in induced PMSCs for 10 days. Scale bar = 50 μm.

DISCUSSION

MSCs, which could be obtained from a wide variety of tissues, has been used to modulate tissue injury and repair through transplantation. To date, clinical trials have proved the validity and security of MSCs-based therapy in chronic lung diseases [15, 16].

Pulmonary -derived MSCs, which share the same characteristics with other MSCs, has also emerged as an attractive alternative for their therapeutic potential in improving lung function and survival. PMSCs therapeutic potential derived from porcine, sheep, mouse and rat, in lung injury treatment has also been revealed. However, application of goose PMSCs is poorly understood. We successfully established applicable methods for isolation, culture, identification and characterization of goose PMSCs in this current study, implicating a therapeutic source cell to treating lung diseases models. There is not a definitive marker vary MSCs from different species. In order to identify and characterize goose PMSCs, several types of markers have been used. In our present study, goose PMSCs have been identified as a population of stem cells which express CD166, CD90, CD44, CD29, CD71 and CD105, but not CD34 and CD45 that resembles PMSCs derived from different species, such as human, porcine, rat and so on, indicating that PMSCs were of mesenchymal origin [17, 18].

The field of MSCs regenerative medicine is expanding rapidly as a promising option for repairing damaged tissues and organs. In addition to their ability to self-renew, MSCs are also capable of multi-differentiation. According to the criteria of the International Society of Cellular Therapy, multipotential of MSCs are defined by capable of differentiating into cells of adipocytes, osteoblasts, chondrocytes in vitro [14]. Thus, our studies tested adipogenic differentiation potential of PMSCs on the basis of the notable Oil-Red-O stain of lipid accumulation and expression of LPL and PPAR-γ markers. We also explored the osteogenic induction in vitro of PMSCs as indicated by notable Alizarin Red staining and well-defined markers COL1A2 and ATF4 expression. Inducing factor dexamethasone may contributes to the maintance of the morphology of induced adipocytes, osteoblasts and its concentration may regulates osteogenic and adipogenic differentiation of PMSCs [19]. In consequence, dexamethasone of different concentrations were utilized for inducing cells in our current study. Follow-up studies found that miR-130a can promoted nodular aggregates and inhibit lipid formation through influence on osteogenic and adipogenic differentiation directions of the MSCs [20]. Further studies are required to understand the role of dexamethasone and miR-130a in this process which may provide a novel prospect for osteoporosis treatment. We also examined he differentiation of PMSCs into chondrogenic ability in vitro and these cells, similar to PMSCs from different species that previously reported, exhibited detectable Alcian Blue staining and expression analysis of relevant genes.

In types of lung epithelial cells, a type II alveolar epithelial cell can be seen as the progenitor cells of the type I alveolar epithelial cell, which reduces surface tension and prevents the alveoli collapse [21-25]. It has now been established that the differentiation of PMSCs into ATII facilitate efficient repair of the alveolar

epithelium in lung diseases [26]. Some studies have also demonstrated Wnt pathway contributes to the differentiation of lung resident MSCs into ATII cells, and brings low risk conferred by exogenous stem cell therapy in lung injuries [27-29]. In our present study, PMSCs could be readily differentiated into type II alveolar epithelial cells in vitro, which might provide a new direction for the targeted therapy of lung diseases through the regulation of cell differentiation.

Taken together, for the first time PMSCs were isolated and cultured from the lungs of 18-19-day-old germ-free goose embryos. The results demonstrated the biological characteristics of mesenchymal-like cells and that these cells, similar to lung MSCs from other species, possess multipotent differentiation potential along the adipogenic, osteogenic, chondrogenic differentiation lineage and epithelial cells. Thus, characterization of goose lung-derived multipotent stem cells will be helpful to understand the repair mechanisms of injured lung tissue in animal model for studying relevant diseases of the respiratory system.

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Conflicts of Interest: The authors have no competing interests.
Ethical approval: Experimental animal procedures, used in our study, were under approval of Care of Experimental Animals Committee of the Bengbu Medical College.

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