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Experimental study on co-culturing adipose-derived stem cells with osteoblasts under different conditions

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Abstract. – OBJECTIVE: To observe whether adipose-derived stem cells (ADSCS), co-cultured with osteoblasts, can differentiate into osteoblasts and, if so, to study the best-induced conditions, with an ultimate goal of repairing bone defects.

MATERIALS AND METHODS: Adipose-derived stem cells and osteoblasts were isolated from New Zealand white rabbits, and co-cultured in media with either 5% or 10% fetal bovine serum, for up to 4 weeks. The morphology of collected cells was examined under a microscope, and histological staining with alkaline phosphatase and alizarin red was carried out after induction for 1, 2, 3 and 4 weeks. Osteogenesis identification, including mRNA expression of type I collagen and osteocalcin, and alkaline phosphatase, was also performed using RT-PCR.

RESULTS: After 7 days of co-culture, some adipose-derived stem cells became round in both groups. After 14 days of co-culture, adipose-derived stem cells were found highly-differentiated, and stained positively with alkaline phosphatase and alizarin red, similar to mature osteoblasts. The mRNA expression of type I collagen and osteocalcin increased in both groups, especially in the 10% fetal bovine serum group.

CONCLUSIONS: Our findings indicate that adipose-derived stem cells co-cultured with osteoblasts can differentiate into osteoblasts when induced by a high concentration of serum culture.

Key Words:
Adipose-derived stem cells, Osteoblasts, Co-culture, Differentiation, Serum concentrations.

Introduction

The repair of bone defect remains a great challenge for clinical orthopedic surgeons. The development of bone tissue engineering has provided a novel solution for the repair of the bone defects. In particular, tissue-engineered bone and the transgenic engineering of bone[1-4] have attracted significant research interest in this field. Adipose-derived stem cells (ADSCs) are adult stem cells characterized by their widespread availability, ease of assimilation, rapid proliferation, multipotential differentiation, and safety. They have become one of the popular cell sources and hot topics in tissue engineering[5]. Studies have shown that co-culturing bone marrow stem cells with osteoblasts could induce bone marrow stem cells to differentiate into osteoblasts. ADSCs had the immunological cell surface marker, the CD molecules, similar to those in bone marrow stem cells (BMSCs), the latter of which have also been widely investigated in the field of tissue engineering. It appeared to have a great possibility that ADSCs could replace BMSCs as a new source of seeding cells in tissue engineering studies. For now, it remains unclear whether osteoblasts have the same effect on ADSCs as BMSCs do. In this study, through co-culturing adipose-derived stem cells with osteoblasts, we attempted to find out whether or not osteoblasts can induce adipose-derived stem cells to differentiate into osteoblasts. Our ultimate goal is to develop a new method for potential bone repair.

Materials and Methods

Approval was obtained from the Ethical Committee of the First Hospital of Xuzhou for this study protocol. Animal handling and care in our experiments were consistent with the regulations in *Guidance suggestion of caring for laboratory animals* stipulated by the Ministry of Science and Technology, the People's Republic of China, 2006[6].

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Materials

Major materials and equipment used in this study were listed in Table I.

Experimental animals: 2 clean healthy New Zealand white rabbits, 3 months old, male or female, weighing 2.5-3.5 kg, were provided by the Experimental Animal Center of Xuzhou Medical College (License No. SCXK (Suzhou) 2005-0005).

Experimental Methods

Collection of ADSC

Rabbit ADSC[7,8] were obtained from two 3-month old New Zealand rabbits. Firstly, 10% chloral hydrate was injected intravenously into the marginal ear vein, at a dose of 2 mL/kg. After anesthetization, the rabbits were secured to an operating table, their fur was shaved around the operating area, and the skin was disinfected. Then, they were surgically draped. An incision was made in the inguinal region and the superficial fascia and deep fascia were bluntly dissected until the inguinal fat pad was completely exposed, which was then removed and put into a 10cm sterile dressing bowl. Under aseptic conditions, the envelope, connective tissues and small blood vessels were cleared off, after which, phosphate buffered saline (PBS) was used to wash 3-5 times, and to remove the red blood cells. Eye scissors were used to cut the tissue into three, 1.0-2.0 mm pieces. The tissue samples were combined with 3-5 times of 0.1% type I collagenase, and the mixture was placed in a 37°C shaking bath for 60 minutes. Dulbecco's modified Eagle medium/F12 (DMEM/F-12) culture medium, consisting of 10% of fetal calf serum (FCS), was added to terminate the process. The mixture was then filtered through a molecular sieve, and centrifuged for 5 minutes, at 1,200 rpm. The supernatant was discarded. DMEM/F-12 culture medium, consisting of 10% fetal calf serum, was added to the precipitate to resuspend cells and the collective concentration of cells was adjusted to 1×10^9 L^{-1}. The cells were then inoculated in culture bottles containing culture medium, and then placed into a homeothermic incubator at saturation humidity. 24 hours later, the culture bottles were gently shaken and observed under a microscope. After most cells attached to the bottle wall, the solution was changed for the first time and the cells that were not attached to the wall were removed. The solution was changed once every three days, and the growth of cells was observed under an inverted phase contrast microscope. After cell fusion had reached 80%-90%, purification was begun and the first cell passage was made.

Identification of Rabbit Stem Cells (ADSCs)9-10

To detect stem cell surface markers, the passage 3 adipose-derived stem cells were collected and inoculated in a 6-well plate pre-covered with a slide. 1.5 mL DMEM/F-12 culture medium, consisting of 10% FCS, was added. The mixture was then placed into a 37°C constant temperature incubator at 5% CO_2 saturation humidity until cell fusion reached 80%-90%. Then, the mixture was washed with PBS three times, for 5 minutes each time, and fixed with 40 g/L paraformaldehyde for 30 minutes. Then, after 3x PBS washes, for 5 minutes each time, 0.2% Triton X-100 was applied, for 2-5 minutes, to permeabilize the mixture. Then once more, the mixture was washed with PBS, 3 times, for 5 minutes each time, blocked with 5% volume fraction of goat

Table I. Major reagents and equipment used in the co-culturing experiment of ADSCs and osteoblasts under various conditions.

Reagents and equipment	Source
DMEM/F-12 culture medium, fetal calf serum	American Thermo Co., Ltd.
Trypsin, type I collagenase, type II collagenase	Beyotime Biotechnology
Rabbit Anti-CD44/CD45	Beijing Bioss Biological technology Co., Ltd.
FITC mark goat rabbit IgG	Beijing ZSGB Biological Technology co., LTD
Alkaline phosphatase assay kit, alkaline phosphatase staining kit	Nanjing Jiancheng Bioengineering Institute
Total RNA extraction kit, RT kit, PCR reaction liquid	Beijing Tian Gen Corporation
Bench-top	Suzhou Purification Equipment Factory
PCR amplifier	American MJ Research Corporation
UV transilluminator	American Alpha Innotech Corporation

serum, and incubated at room temperature for 30 minutes. The confining solution was disposed of, and the primary antibody, the rabbit anti-mouse CD44, CD45 antibody response solution was added. The mixture was incubated overnight at 4°C. Next, the primary antibody was disposed of and the mixture was washed with PBS, 3 times, for 5 minutes each time. The second antibody, labeled with FITC fluorescein, was added and the mixture was incubated at 37°C in a dark environment for 30 minutes, followed by 3 washes with PBS, 5 minutes each time, and fixed with 95% glycerinum. Finally, the developed staining was observed under a fluorescence microscope.

Extraction, Culture, and Passage of Rabbit Osteoblasts

3-month old New Zealand rabbits were injected intravenously with 10% of chloral hydrate at a dose of 2 mL/kg, through the ear marginal. After anesthetization and disinfection, the rabbit skulls were separated from their bodies and extracted, in a sterile environment, and placed separately into culture dishes pre-filled with PBS. Impurities, the periosteum, connective tissue and blood vessels were removed, and the skulls were washed with PBS 3 times. 1 mm×1 mm pieces were cut from the skulls and transferred to a 15 ml centrifuge tube, where 0.25% pancreatin was added, and left to process in a water bath at 37°C and cultured in a 5% CO_2 incubator with saturation humidity. Once the osteoblasts began adhering to the culture bottle wall, the solution was washed twice with PBS, after which the cells that were not attached to the wall were removed and a complete medium was added to the culture. The medium was changed once every three days. On the 6th day, the osteocommas were removed and culturing continued. Once the cells had spread over 80% of the culture bottle, serial subcultivation was begun.

Co-culture of Osteoblasts and Adipose-derived Stem Cells

Two co-culture groups, the 10% FBS co-culture group and the 5% FBS co-culture group, were tested in our study. In both groups, the osteoblasts and adipose-derived stem cells were combined together at a ratio of 1:1 with a final inoculating concentration of $5.0x10^7$ cells/mL. The osteoblasts and adipose-derived stem cells were cultivated, respectively, in a 6-well plate and a 25 ml culture bottle. The culture medium was changed every two days, and the morphologic changes of adipose-derived stem cells were observed under an inverted phase contrast microscope. After 7 days and 14 days of culturing, pancreatin was used to terminate the digestion process. Then the co-cultured adipose-derived stem cells were implanted into culture bottles and culture dishes for plate creeping and RNA extraction.

Alkaline Phosphatase Staining and Alkaline Phosphate Test

After 2 weeks of induction, the glass covers of both 6-well plates were removed and standard operating procedures were followed according to alkaline phosphatase staining kit specifications. To measure the concentration of alkaline phosphate, the cells were collected centrifugally after 1 week, 2 weeks, 3 weeks and 4 weeks of induction, and put into a 96-well plate. 0.2 mL 1% TritonX-100 was added and the mixture was left overnight at 4°C, and then treated in accordance with the specifications of the alkaline phosphatase test.

Alizarin Red Staining

After 2 weeks of induction, the glass covers of both 6-well plates were removed and the culture was fixed for 30 minutes using paraformaldehyde (40 g/L). After PBS wash, 1 ml 2% alizarin red staining solution was applied. The cells were then incubated at room temperature for 20 minutes, followed by microscopic observation and photo documentation under an inverted phase contrast microscope equipped with a digital camera.

mRNA Expression of Osteocalcin and Type I Collagen under RT-PCR Detection

After osteogenic induction for 7 and 14 days, 55 mmol/L sodium citrate was used to dissolve the composite support. The cells were then separated and collected centrifugally. Trizol reagent was used to split the cells, extract complete RNA and to perform a purity identification. An RT kit was then used to inversely transcript RNA into cDNA and, finally, to augment PCR. Primer design was as follows: 1) osteocalcin: upstream: 5'-CATGAGAGCCCTCACA-3' downstream: 5'-TCAAGGAAGGGCAAACG-3; 2) type I collagen: upstream: 5'-GGCAAACATGGAAACCG-3 downstream: 5'-TCAAGGAAGGGCAAACG-3'; β-actin upstream: 5'-GTGGGGCGCC-CCAGGCACCA-3' downstream: 5'-CTTCCT-TAATGTCACGCACGATTTC-3'.

PCR conditions: samples were pre-degenerated at 94°C for 5 minutes, then degenerated at

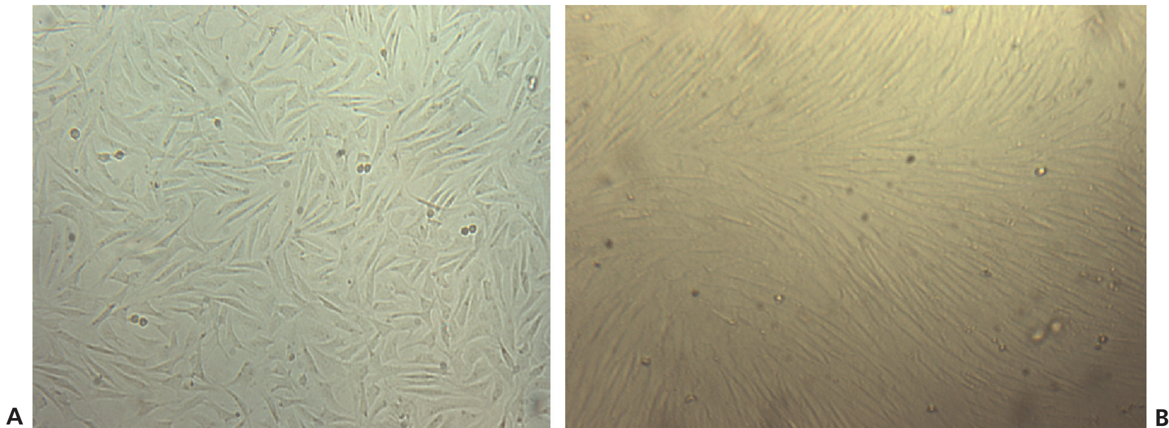

Figure 1. Morphology of primary ADSCs ***(A)*** and Passage 3 ADSCs ***(B)***. After the culture medium was changed for the first time, the majority of adherent cells were of a fusiform, polygonal shape (A). Passage 3 cells, though still in a typical long fusiform, grew in a spiral pattern ***(B)***. Images were originally taken at 100x.

94°C for 30 seconds, annealed at 52°C for 30 seconds, then extend at 72°C for 1 minute. Continued such circulation for 30 times and then extended at 2°C for 10 minutes. The PCR products were scanned to detect the electro-fluorescence and the absorbance of DNA, and the ratio was determined by reference to the β-actin stripe, which indicates the level of mRNA expression.

Major observation index: 1) morphological changes of adipose-derived stem cells were observed and the immunofluorescent staining method was used to detect the expression of CD44 and CD45 on the surface of adipose-derived stem cells. 2) morphological observation after the co-culture of osteoblasts and adipose-derived stem cells. 3) Alkaline phosphatase and alizarin red staining were used to detect the osteoinductive differentiation of adipose-derived stem cells. 4) mRNA expression of type I collagenase and 1w, 2w cell osteocalcin. 5) the activity of osteoblast alkaline phosphatase.

Statistical Analysis

The SPSS 13 statistical package (SPSS Inc., Chicago, IL, USA) was applied to perform statistical analysis. Data were presented as x±s. The *t*-test was applied to make comparisons between the two groups, and single factor variance analysis was applied to determine the significance. The Student-Newman-Keuls SNK test was used to make pair-wise comparisons. $p < 0.05$ was considered statistically significant.

Results

Isolation and Identification of ADSCs

After initial isolation, cells were small and of a rounded shape. After being cultured for 24 hours, they began to adhere to the culture bottle wall. The non-adhered cells were elliptocytes, and the majority of adherent cells were of a fusiform, polygonal shape (Figure 1A). After the first change of solution, the non-adhered cells were removed. As culture grew, the number of cells increased and cells became typically of a long, fusiform shape that grew into groups and clusters. After cell fusion had reached 80%-90%, cells developed into the next generation. Passage 3 cells grew in a spiral shape that maintained long-term culture stability (Figure 1B). Immunofluorescent staining was applied to detect ADSC surface molecules. Our results showed that CD44 immunofluorescent staining was positive, indicating that the cells were mesenchymal stem cells. On the other hand, CD45 immunofluorescent staining was negative, confirming that the detected cells had not originated from stem cells belonging to the circulatory blood (Figure 2). Therefore, we concluded that the isolated cells were ADSCs.

Cell Morphological Changes After Co-culture

After co-culture for 7 days, certain ADSCs became polygonal, rounded and anomalous; the multiplication rate slowed down and the change in the 10% FBS group was obvious (Figure 3). After co-culture for 14 days, morphological features of ADSCs of the 10% FBS co-culture

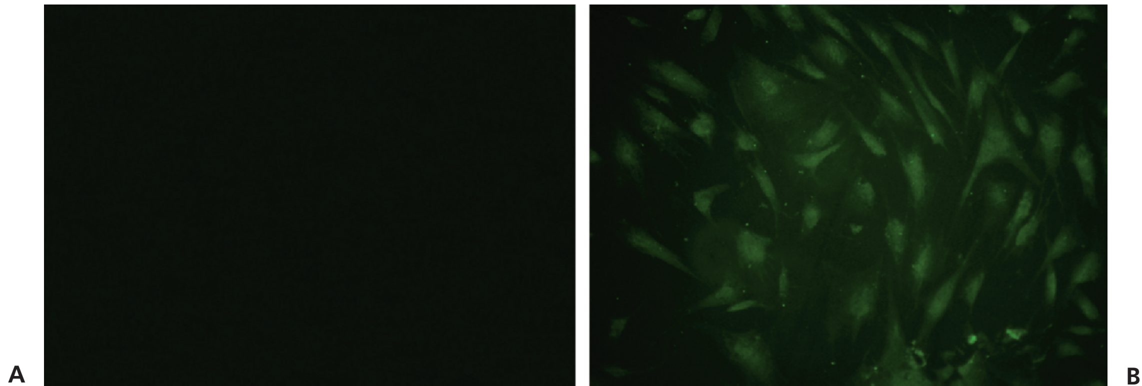

Figure 2. Immunofluorescent staining of ADSCs identification. ***A,*** negative CD45 expression confirming that the detected cells were not originated from the stem cells belonging to the circulatory blood. ***B,*** positive CD44 expression signifying mesenchymal stem cells. Original images were taken at 100x.

group were similar to osteoblasts. In the 5% FBS co-culture group, there was also high differentiation of the cells, though with an aging tendency (Figure 4).

Alkaline Phosphate Staining and Alizarin Red Staining

After osteogenic induction for 14 days, alkaline phosphate staining was positive in both groups. A large number of cell nuclei showed dark blue staining and the endochylema showed black/blue staining, as observed under an inverted phase contrast microscope. There was no significant difference between the two groups. But we found a higher number of positive cells in the 10% FBS co-culture group versus the 5% co-culture group. Similarly, after osteogenic induction for 14 days, alizarin red staining was positive in both groups and orange circular or oval calcium nodules could be seen under the microscope. There was no significant difference between the two groups. But the number and the size of calcium nodules in the 10% FBS co-culture group were much larger than those in the 5% FBS co-culture group (Figure 5).

Detection of the Activity of Alkaline Phosphatase

After 1 week, 2 weeks, 3 weeks and 4 weeks of induction, the concentration of alkaline phosphate produced by the ADSCs was measured. As listed in Table II, the experimental group generated significantly more alkaline phosphatase than the control group at all tested time points. In par-

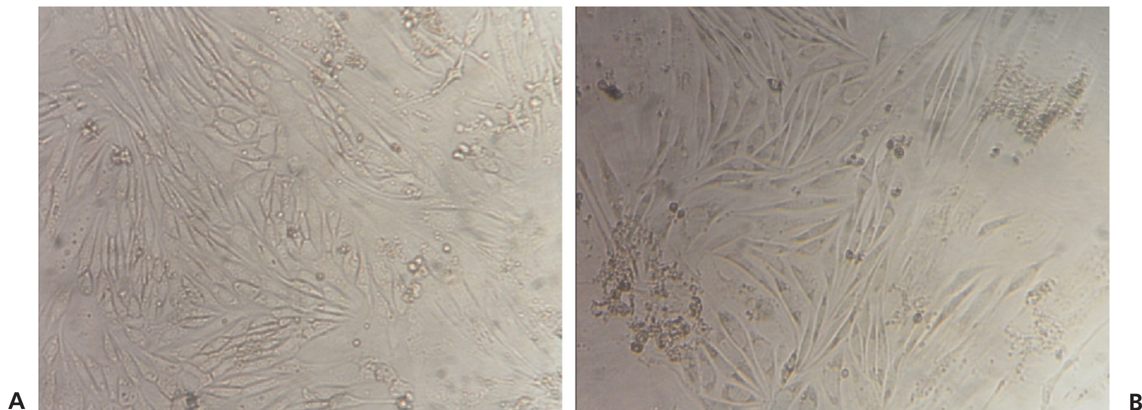

Figure 3. Observation on morphological changes of ADSCs from the 10%FBS co-culture group (A) and the 5%FBS co-culture group (B) following 1-week osteogenic induction. Certain ADSCs became polygonal, rounded and anomalous, and the multiplication rate slowed down. The morphological change of ADSCs in the 10% FBS group (A) was more obvious compared to the 5%FBS group (B). Original images were taken at 100x.

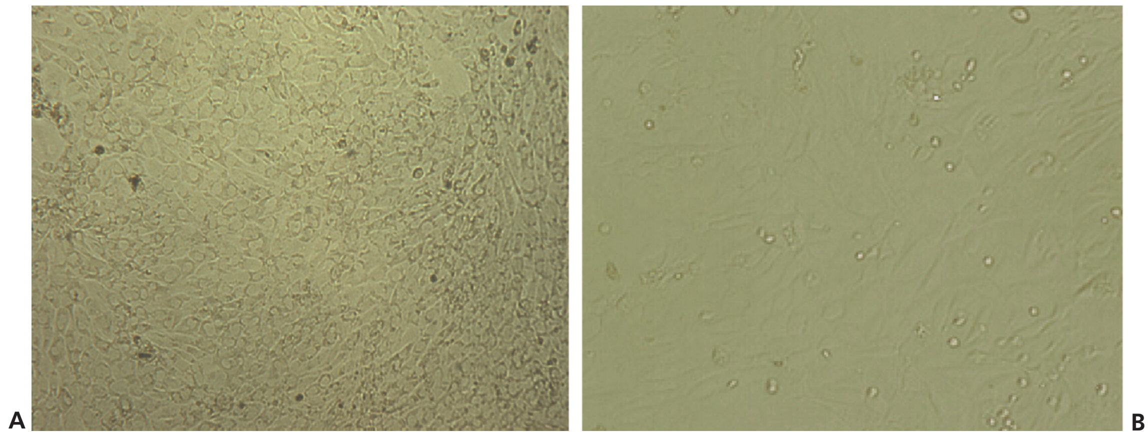

Figure 4. Observation on morphological changes of ADSCs from the 10%FBS co-culture group (A) and the 5%FBS co-culture group (B) following 1-week osteogenic induction. Certain ADSCs became polygonal, rounded and anomalous, and the multiplication rate slowed down. The morphological change of ADSCs in the 10% FBS group (A) was more obvious compared to the 5%FBS group (B). Original images were taken at 100x.

ticularly, the detected alkaline phosphatase concentration reached an apex after 2 weeks of osteogenic induction, which was more than 3-folds that in the non-induction control group.

mRNA Expression of Type I Collagen and Osteocalcin by RT-PCR Detection

Results from RT-PCR detection showed that after osteogenic induction for 7 days and 14 days, expressions of type I collagen and osteocalcin were found in cells from both groups, and increased as time elapsed. According to the observed gray levels, mRNA expression of both type I collagen and osteocalcin in the 10% FBS co-culture group were stronger than those in the 5% FBS co-culture group. mRNA expressions of type I collagen were more abundant as observed by product expression. At the same time point, both the mRNA expression of type I collagen and osteocalcin, in the experimental group, were much stronger that those in the control group (Figure 6).

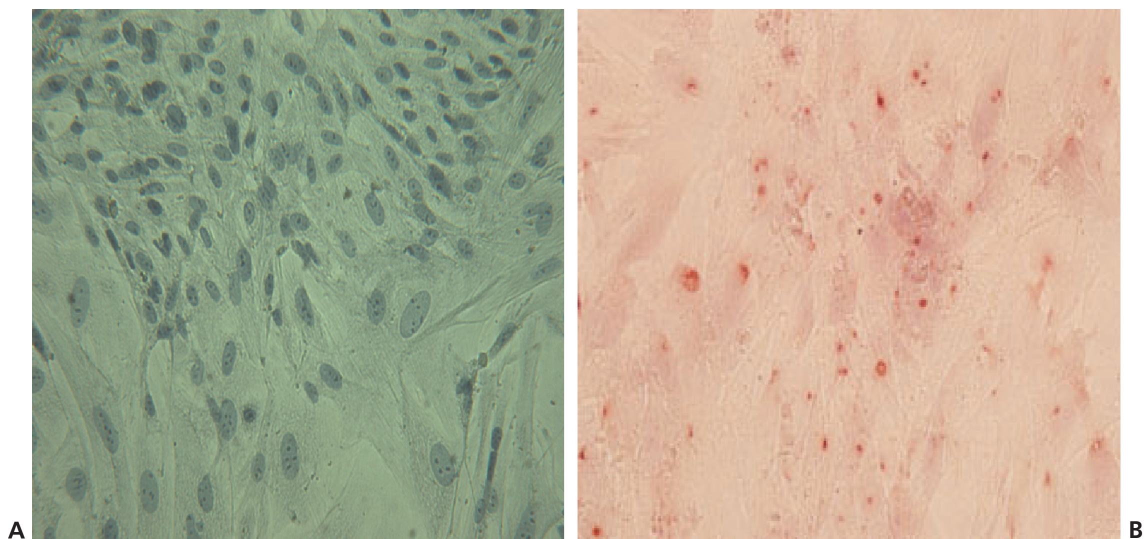

Figure 5. Observation on morphological changes of ADSCs from the 10%FBS co-culture group *(A)* and the 5%FBS co-culture group *(B)* following 2-week osteogenic induction. High differentiation of ADSCs in the 10% FBS co-culture group was similar to osteoblasts. In the 5% FBS co-culture group, high differentiation was also seen, but the cells tended to aging. Original images were taken at 100x.

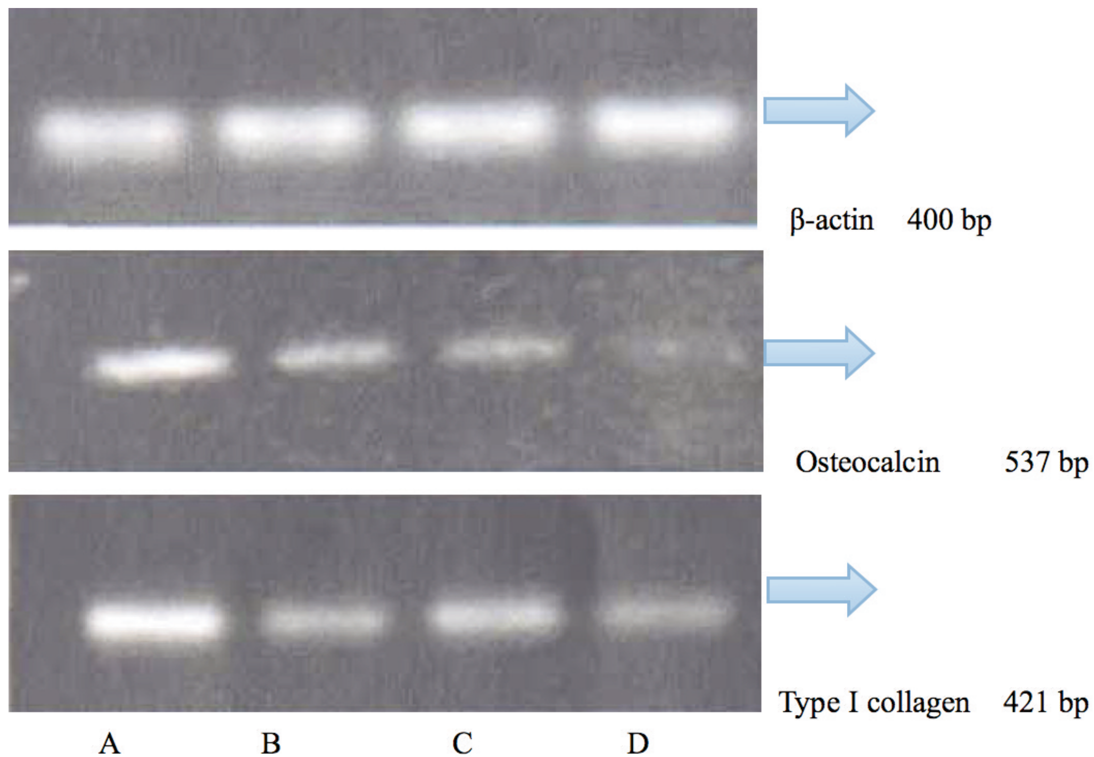

Figure 6. Type I collagen and mRNA expression of osteocalcin under RT-PCR detection in cells from both groups. ***A,*** the 5% FBS co-culture group after 1-week induction. ***B,*** the 10%FBS co-culture group after 1-week induction. ***C,*** the 5% FBS co-culture group after 2-week induction. ***D,*** the 10%FBS co-culture group after 2-week induction. After co-culture for 7 days and 14 days, mRNA expression of both type I collagen and osteocalcin in the 10% FBS co-culture group were stronger than those in the 5% FBS co-culture group, indicating that ADSCs and osteoblasts, after co-culture with a higher concentration of serum, could promote more ADSCs to differentiate into osteoblasts.

Discussion

The repair of bone defects, resulting from traumas or tumors, has always been a challenging task for clinical orthopedic surgeons. Conventional treatment methods, including transplantation of autogenous bones, allogeneic bone and artificial bone, achieved different degrees of efficacy but have limitations. For example, autogenous bones are few, and bone removal causes extra injury and pain to the patients; the use of allogeneic bone and artificial bones may have antigenic problems and can cause immunological rejection, which can result in reconstruction failures. Furthermore, there are also risks of cross infections and virus infections, thus limiting their

Table II. Detected alkaline phosphatase concentrations at different periods of ADSCs osteogenic induction (King unit/100 ml, mean±sd, n=4).

Group	1-week	2-week	3-week	4-week
Experimental	0.8121± 0.098	1.6816±0.1433	1.0766± 0.3779	0.8790±0.1170
Control	0.3514±0.0339	0.5765± 0.0740	0.4306±0.1473	0.3876±0.0557
t-value	16.354	13.641	4.639	6.154
p-value*	0.0002	0.001	0.011	0.007

*$p<0.05$ comparing the experimental calcitonin gene related peptide (CGRP) induction group to the non-induction control group; #$p<0.05$ comparing within the CGRP induction group at different induction time points

applications[11]. Development of bone tissue engineering provides a new solution to the repair of bone defects. Bone tissue engineering applies the principles and methods of tissue engineering to the research and development of bone substitutes that could repair bone defects and promote bone regeneration. Bone substitutes could avoid the shortcomings of conventional treatment methods and also have very good biological compatibility, sufficient mechanical capacity and biological stability. Artificial bone produced by bone tissue engineering technology could not only repair a large number of bone defects, but also provide customized, large-scale preparation. It is thus a favorable bone substitute material for trauma repair and functional reconstruction.

In the last years, the development of artificial bone has made some progress, but there is still a lack of a bone substitute that is mature in both structure and performance. Studies on bone repair using tissue-engineered and transgenically engineered bones also have achieved varied degrees of success[12-15]. Currently, bone tissue engineering is focused on the study of bone marrow stem cells. Since bone marrow stem cells are hard to obtain and cannot meet the needs of experimental research and clinical applications, some scholars have begun to explore ADSCs, also originated from mesoblast, and investigate whether ADSCs have the same multiple differentiation potentials as bone marrow stem cells do. As a new kind of seed cell, the biological characteristics of ADSCs are quite similar to those of BMSCs[16]. Zuk et al[17] have obtained mesenchymal stem cells, having multiple differentiation potentials, from human adipose tissues and deemed that mesenchymal stem cells can differentiate into osteoblasts, cartilage cells, and fibroblasts. Besides, compared with BMSCs, ADSCs also have the following advantages: (1) abundant resources, easy attainability, slight damages; (2) strong adherent ability and can be easily cultured *in vitro*; (3) simple culturing conditions, rapid expansion *in vitro*; (4) rich in stem cells and high efficiency in attainability; (5) stable phenotype, slow aging, and high proliferative activity; (6) free from any ethical or moral issues[18]. Therefore, there is a great possibility that ADSCs could replace BMSCs and become a new source of seed cells in tissue engineering. Seed cells, induced factor, and support carrier are important elements in tissue engineering technology. ADSCs are a new type of seed cells and have become the focus of bone and soft tissue engineering studies. The advantage of co-culture lies in that it can promote ADSCs to differentiate into osteoblasts without inducing factors. This method is more economical and effective.

In our study, we have applied the immunofluorescent staining method to detect the surface molecules of ADSCs. We found that cell surface antigens were positive, indicating that these kinds of cells were mesenchymal stem cells; while negative CD45 expression confirmed that the detected cells had not originated from the stem cells in the circulatory blood. Therefore, we concluded that the isolated cells were ADSCs.

Osteoblasts could compound and secrete bone matrix and promote the bone matrix to mineralize and form bone tissues. Calcium ions would deposit on collagen under the effect of alkaline phosphatase, which was secreted from osteoblasts, and completes the mineralization of the matrix. Therefore, the detection of alkaline phosphatase is widely used as the major index for identifying and evaluating the functional state of osteoblasts. After co-culture and induction for 2 weeks, the alkaline phosphatase and calcific nodules in both groups were positive. Through an analysis of the changes of alkaline phosphatase concentration and calcium ion concentration, after induction for 1 week, 2 weeks, 3 weeks and 4 weeks, we found that alkaline phosphatase was on an increasing trend in the first two weeks, but gradually decreased after that. Also, we found that calcium ions were on a constantly increasing trend in the 4 weeks and that calcitonin gene-related peptide, in the induction group, was higher than that in the control group. Compared with the control group, the size and number of calcific nodules in the experimental group were greater. After induction for 7 days and 14 days, mRNA expression of both type I collagen and osteocalcin, under RT-PCR detection, in the experimental group were higher than those in the control group, thus confirming that ADSCs had realized induced differentiation into osteoblasts and that osteoblasts had the effect of promoting ADSCs to differentiate into osteoblasts. Calcification of the extracellular matrix was a characteristic sign, which signified the differentiation of the osteoblasts in the later period. Under *in vitro* culture, calcification of osteoblasts was an important basis for the identification of osteoblast. Alizarin red staining was the major method used to observe the formation of calcium nodules *in vitro*. The stain would chelate with calcium ions in nodules and display the location that calcium deposited. The formation of ALP was positively correlated with

the differentiation and maturity of osteoblasts. Type I collagen was one of the major components of the bone matrix, which could phosphorylate target protein through protein kinase and increase the synthesis of cell proteins. Besides, it could also activate Na^+/H^+ exchange system, promote the adherence, proliferation and differentiation of osteoblasts as well as the formation of new bones[19,20]. Osteocalcin was compounded and secreted by osteoblasts. It was a special and sensitive marker that reflected the activity of osteoblasts. Some studies have found that osteocalcin improves carbohydrate metabolism, which might also be one of the reasons that bone repair became a high-energy process[21].

Conclusions

In this experimental study we successfully co-cultured osteoblasts and ADSCs, and induced rabbit ADSCs to differentiate into osteoblasts. Further *in vitro* and animal studies are required to investigate how these ADSCs-induced osteoblasts grow in a support carrier and whether they could survive *in vivo*.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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