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嚴重再生不良性貧血病童之骨髓間質幹細胞研究：其增生與分化的潛能較差

Poor potential of proliferation and differentiation in bone marrow mesenchymal stem cells derived from children with severe aplastic anemia

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中文摘要

研究目的

特發性嚴重再生不良性貧血（idiopathic severe aplastic anemia; SAA）雖然罕見，但對病童卻有著生命的威脅；其發病的原因主要是喪失骨髓造血的功能，但直至今日真正的致病機轉卻還未能瞭解透徹。骨髓間質幹細胞（mesenchymal stem cells; MSCs）在骨髓造血微環境中扮演舉足輕重的角色，可提供造血幹細胞（hematopoietic stem cells）生長分化成熟之所需。本研究的目的為探討嚴重再生不良性貧血病童之骨髓間質幹細胞基本特質的缺陷。

研究方法

分別自五位嚴重再生不良性貧血的病童及五位對照組兒童取得骨髓抽出液，再將間質幹細胞分離並作體外培養。藉由觀察骨髓間質幹細胞之細胞型態（morphology）、測定其免疫表面標誌的表現（immunophenotyping）、分析其增生能力（proliferative capacity）和分化潛能（differentiation potential），比較兩組之間是否有差異。

研究結果

雖然嚴重再生不良性貧血病童及對照組兒童兩組之骨髓間質幹細胞體外培養的細胞型態均呈現相似之紡錘狀，且免疫表面標誌的表現均為 CD45、CD14、CD34 陰性和 CD105、CD73、CD44 陽性；但增生能力的實驗發現，嚴重再生不良性貧血病童之骨髓間質幹細胞的增生速度較慢且累積群體倍增殖（cumulative population doubling）較對照組小，分別為 1.83 ± 1.21 與 3.36 ± 0.87（p = 0.046），這意味著嚴重再生不良性貧血病童的骨髓間質幹細胞有著較低的增生能力。經過骨分化誘導（osteogenic induction）之後，嚴重再生不良性貧血病童之骨髓間質幹細胞表現較弱的鹼性磷酸塩酶活性（alkaline phosphatase activity；1.46 ± 0.04 與 2.27 ± 0.32；p = 0.013）、呈現較淡的 von Kossa 染色和較低的 core binding factor α1 基因表現（0.0015 ± 0.0005 與 0.0056 ± 0.0017；p = 0.013）。經過脂肪分化誘導（adipogenic induction）之後，嚴重再生不良性貧血病童之骨髓間質幹細胞呈現較淡染的 Oil red O 染色（0.86 ± 0.22 與 1.73 ± 0.42；p = 0.013）和較低的 lipoproteinlipase 基因表現（0.0105 ± 0.0074 與 0.0527 ± 0.0254；p = 0.013）。 以 real time-PCR 分析骨分化與脂肪分化之特定基因表現的結果和特殊染色的發現一致，兩者都代表著嚴重再生不良性貧血病童之骨髓

間質幹細胞骨分化與脂肪分化的潛能較差。

研究結論

本研究中，我們首次成功地顯示：嚴重再生不良性貧血病童之骨髓間質幹細胞的增生與分化潛能較差。而骨髓間質幹細胞的改變或許和骨髓造血能力的喪失有著密切的相關，這需要後續的研究進一步來闡明骨髓間質幹細胞與嚴重再生不良性貧血兩者之間的相關性。

英文摘要

Background

Idiopathic severe aplastic anemia (SAA), characterized by failure of hematopoiesis, is rare and potentially life-threatening to children. However, the pathogenesis has not been completely understood, and insufficiency in the hematopoietic microenvironment can be an important factor. Mesenchymal stem cells (MSCs) play an important role in maintaining bone marrow microenvironment. Therefore, we aimed at the intrinsic defects of bone marrow MSCs derived from SAA children.

Materials and Methods

Bone marrow MSCs were obtained from 5 SAA children and 5 controls. The morphology, immunophenotyping, proliferative capacity and differentiation potential of MSCs from SAA children were determined and compared with those of MSCs from controls.

Results

MSCs of SAA and control group shared a similar spindle-shaped morphology in vitro. Both revealed a consistent immunophenotypic

profile which was negative for CD45, CD14 and CD34, and positive for CD105, CD73, and CD44. However, SAA MSCs had slower expansion rate and smaller cumulative population doubling from passage 4 to 6 (1.83 ± 1.21 vs 3.36 ± 0.87; p = 0.046), indicating lower proliferative capacity. Besides, only 3 of 5 cultures of SAA group retained the ability to continue expansion till 80%-90% confluent cell layer beyond passage 6, suggesting earlier senescence of SAA MSCs. After osteogenic induction, SAA MSCs showed lower alkaline phosphatase activity (1.46 ± 0.04 vs 2.27 ± 0.32; p = 0.013), less intense von Kossa staining and lower gene expression of core binding factor α1 (0.0015 ± 0.0005 vs 0.0056 ± 0.0017; p = 0.013). Following adipogenic induction, SAA MSCs showed less intense Oil red O staining (0.86 ± 0.22 vs 1.73 ± 0.42; p = 0.013) and lower lipoproteinlipase expression (0.0105 ± 0.0074 vs 0.0527 ± 0.0254; p = 0.013). The results of real time-PCR analysis for the assessment of lineage-specific genes were consistent with the findings of histochemical stains, and both indicated that SAA MSCs had poor osteogenic and adipogenic potential.

Conclusions

In this study, we demonstrated that bone marrow MSCs from children with SAA had poor potential of proliferation and differentiation. These alterations in MSCs may contribute to the failure of hematopoiesis, and lead to the development of the disease. Further studies are needed to elucidate the relationship between MSCs and SAA.

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目錄

圖目錄

第一章 前言

1.1 研究背景

Childhood acquired aplastic anemia, characterized by failure of hematopoiesis, is rare and potentially life-threatening with an annual incidence of 1-6 per million [1-3]. Severe aplastic anemia (SAA) is defined as profound bone marrow (BM) hypocellularity and marked peripheral blood pancytopenia. Despite of many putative etiologies, a specific cause cannot be identified in most children, and is termed "idiopathic SAA". Significant advances have been made in the management of the disease, including allogeneic stem cell transplantation and immunosuppressive therapy [1, 3, 4]. However, the mechanism by which idiopathic SAA develops has not been completely elucidated. Although many studies demonstrated the association of immune-mediated pathogenesis, up to 30% of patients do not have detectable evidence for an underlying immune basis and not respond to immunosuppressive therapy [1]. Therefore, other mechanisms do exist.

Mesenchymal stem cells (MSCs), first described by Friedenstein *et al.* [5], have the capacity of self-renewal and differentiation into mesenchyme-lineage cells. BM MSCs can interact with hematopoietic stem cells (HSCs) and secret cytokines and regulatory molecules [6-11].

They play a crucial role in providing a specialized microenvironment for HSC survival and differentiation [6, 7, 9-11]. Therefore, MSC dysfunction may result in the impairment of hematopoiesis, and lead to the development of SAA.

1.2 研究目的

However, data focusing on the role of MSCs in the pathophysiology of SAA are very limited [12-14]. Till now, no information about the characteristics of morphology, immunophenotyping, proliferative capacity and differentiation potential of SAA MSCs has been reported. In order to clarify the pathophysiology of SAA and to identify the characteristic changes of SAA MSCs, we compared these basic properties of BM MSCs derived from SAA children and controls.

第二章 研究方法

2.1 研究材料

BM cells were obtained from iliac crest aspirates. Idiopathic SAA was defined as pancytopenia and hypocellular BM after excluding any other underlying diseases. To diagnose SAA, BM cellularity of less than 25% and at least two of the following criteria must be fulfilled: absolute neutrophil count less than 0.5×10^9/L, platelet count less than 20×10^9/L, and reticulocyte less than 1% [1, 3, 15]. Control subjects were patients who received BM examination for diseases other than hematological diseases with pathological proof of normal BM. All patients were previously untreated and aged less than 18 years old. The institutional review board of Tungs' Taichung MetroHarbor Hospital approved this protocol, and written informed consents were obtained from the parents or legal guardians of the patients.

2.2 研究設計

2.2.1 Cell culture

Mononuclear cells were isolated from BM aspirates by Ficoll-Paque density centrifugation (1.077 g/ml; Amersham Biosciences, Uppsala, Sweden) and then seeded in low-glucose Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic-Antimycotic (Gibco). Cells were incubated at 37°C with 5% CO_2 in a humidified atmosphere. After 48 hours, medium with suspension of nonadherent cells was discarded and fresh medium was added. Thereafter medium was replaced twice a week. When reaching 80%-90% confluence, cells were detached with 0.25% trypsin-EDTA (Gibco) and replated at a concentration of $8.5 \times 10^3/cm^2$ in 10-cm dishes.

2.2.2 Immunophenotypic analysis

Cultured MSCs (passage 4) were detached, washed, and resuspended in phosphate-buffered saline (Gibco). After fixing and blocking, the cells were immunolabeled with the following mouse anti-human antibodies: fluorescein isothiocyanate-conjugated CD45 (FITC-CD45; BD Biosciences, San Jose, CA), phycoerythrin-conjugated CD14 (PE-CD14; BD Biosciences), FITC-CD34 (BD Biosciences), FITC-CD105 (Serotec, Oxford, UK), PE-CD73 (BD Pharmigen, San Diego, CA) and FITC-CD44 (BD Pharmigen). The nonspecific mouse IgG (BD Biosciences) served as isotype control. Data were analyzed by flow cytometry (FACSCalibur; BD Biosciences) with CellQuest software.

2.2.3 Proliferative capacity

Yield of cells at each passage was enumerated using Trypan blue (Gibco) to exclude dead cells. The population doubling (PD) of cultured MSCs was calculated according to the equation: PD = $\log_2$ (the number of viable cells at harvest / the number of seeded cells). The cumulative PD was the sum of PD from passage 4 to 6.

2.2.4 Osteogenic and adipogenic potential

To evaluate differentiation potential, cultured MSCs (third passage, at 80%-90% confluence) were subjected to osteogenic and adipogenic differentiation *in vitro*. Cells were detached from culture dishes and replated in 60-mm dishes for further studies.

To promote osteogenic differentiation, cells were incubated in DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma, St Louis, MO), 0.1 μM dexamethasone (Sigma) and 0.2 mM ascorbic acid (Sigma) for 3 weeks. On day 21, cultures were stained for alkaline phosphatase (ALP; Sigma) activity and mineralized deposits were detected by von Kossa stain (Cedarlane, Ontario, Canada). To quantify ALP activity, 2 ml of 0.05 N NaOH in ethanol was added to each dish after ALP activity stain and the extraction was measured by spectrophotometry (Ultrospec 1100 pro; Amersham Biosciences) at 550 nm.

For induction of adipogenic differentiation, cells were grown in DMEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 0.1 mM indomethacin (Sigma) and 10 μg/ml insulin (Novo Nordisk A/S, Bagsværd, Denmark) for 2 weeks. On day 14, adipogenic differentiation was demonstrated by intracellular accumulation of lipid droplets stainable with oil red O

(Sigma). The dye content was eluted by ethanol and quantified spectrophotometrically.

Differentiation was verified further by real-time polymerase chain reaction (Q-PCR) for the assessment of lineage-specific genes as core binding factor α1 (Cbfa1) for osteocytes and lipoprotein lipase for adipocytes. MSCs cultured in osteogenic and adipogenic induction medium were harvested on day 21 and 14, respectively. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Concentration of the RNA samples was estimated spectrophotometrically at OD 260/280, and cDNA was synthesized using MMLV reverse transcriptase (Epicentre Biotechnologies, Madison, WI) in the presence of oligo-dT primer (Promega, Madison, WI). The sequences of PCR primers were as follows: Cbfa1 [16], sense 5'-CATGGCGGGTAACGATGAA-3' and antisense 5'-CGGCCCACAAATCTCAGATC-3'; lipoprotein lipase [17], sense 5'-ATGGAGAGCAAAGCCCTGCTC-3' and antisense 5'-TACAGGGCGGCCACAAGTTTT-3'. The expression of β-actin (sense 5'-TGTGGATCAGCAAGCAGGAGTA-3' and antisense 5'-CAAGAAAGGGTGTAACGCAACTAAG-3') was used as an internal control to normalized specific gene expression in each sample [16]. Q-PCR was performed using cDNA samples with SYBR Green

PCR master mix (Applied Biosystems, Foster City, CA) and carried out in the ABI 7300 Real-Time PCR system (Applied Biosystems).

2.3 統計方法

Data analysis was performed using SPSS 14.0 for Windows. Results are presented as mean ± standard deviation and 95% confidence interval. Kolmogorov-Smirnov Z test was used for comparison of the two groups. Statistical value of $p < 0.05$ was considered significant.

第三章 研究結果

3.1 Morphology and immunophenotypic profile

A total of 10 children, 5 SAA patients and 5 controls, were enrolled in the study. The average age was 11.9 and 11.6 years old, respectively. *In vitro*, MSCs of SAA and control group shared a similar spindle-shaped morphology (Fig. 1A). Both revealed a consistent immunophenotypic profile which was negative for CD45, CD14 and CD34, and positive for CD105, CD73, and CD44 (Fig. 1B). No significant difference was noted in the expression of any single surface marker between the two groups.

3.2 Proliferative capacity

To prevent hematopoietic cell contamination, which might be present in earlier passages, or the presence of senescent or differentiating MSCs in later passages, we used cells from passage 4 to 6 for the study of growth kinetics. SAA group had slower expansion rate than control group, shown as average PD of each passage (Fig. 2A). Two of five cultures of SAA group stopped proliferating at passage 5 and 6, respectively; whereas all cultures of control group continued to grow well. Lower proliferation potential of SAA MSCs was also demonstrated by smaller cumulative PD (1.83 ± 1.21 vs 3.36 ± 0.87; $p = 0.046$) (Fig. 2B).

3.3 Differentiation potential

When exposed to osteogenic induction medium, MSCs from SAA children had less robust osteogenic differentiation than MSCs from controls as shown by lower ALP activity (Fig. 3A). Greater extent of mineralization in control group was also demonstrated by more intense von Kossa stain (Fig. 3B). Under adipogenic condition, SAA MSCs gave rise to less lipid-containing cells. The intracytoplasmic vacuoles of neutral fat can be identified by Oil red O stain (Fig. 3C). The droplets of fat were more and larger within a single adipocyte in control group. Quantitation of dye content of ALP activity stain in the osteogenic cultures (1.46 ± 0.04 vs 2.27 ± 0.32; $p = 0.013$) and Oil red O stain in the adipogenic cultures (0.86 ± 0.22 vs 1.73 ± 0.42; $p = 0.013$) confirmed the histochemical observations (Fig. 4).

Results of Q-PCR analysis presented in Figure 5 show the assessment of lineage-specific genes of osteogenesis and adipogenesis. After osteogenic induction, MSCs of SAA group expressed lower level of Cbfa1 than MSCs of control group (0.0015 ± 0.0005 vs 0.0056 ± 0.0017; $p = 0.013$). Following adipogenic induction, SAA MSCs showed lower lipoproteinlipase expression (0.0105 ± 0.0074 vs 0.0527 ± 0.0254; $p = 0.013$). The results of Q-PCR analysis were

consistent with histochemical findings, and both strongly suggested that BM MSCs from SAA children had poor osteogenic and adipogenic potential.

第四章 討論

4.1 結果討論

MSCs can be characterized by a panel of surface markers, by their *in vitro* growth pattern and subsequent expansion and by their multilineage differentiation potential [10, 11, 18]. Many studies used these basic properties as indicators to identify MSCs from origins other than BM [8, 16, 17, 19-27]. We aimed at these properties of BM MSCs from SAA children and found that SAA MSCs had poor potential of proliferation and differentiation. The alterations in the characteristics of MSCs may contribute to failure of hematopoiesis and lead to the development of the disease.

Although the morphology and surface marker expression of cultured MSCs did not change, SAA MSCs had slower expansion rate and smaller cumulative PD, indicating lower proliferation potential than normal MSCs. Besides, only 3 of 5 cultures of SAA group retained the ability to continue expansion till 80%-90% confluent cell layer beyond passage 6, suggesting earlier senescence of SAA MSCs. We can not answer the aging mechanisms here. However the heterogeneity in pathogenesis of the disease seems to explain the variations in MSC expansion potential.

The significant decrease in osteogenic and adipogenic potential of SAA MSCs was demonstrated in our study. Even the 3 cultures which can proliferate beyond passage 6 showed lower expression of lineage-specific genes after differentiation under permissive conditions. The results of histochemical stains supported the gene expression study. These findings provided strong evidence that defects in BM MSCs of SAA children do exist.

4.2 其他相關性討論

The immune-mediated HSC destruction for the pathogenesis of idiopathic SAA has been widely accepted and many studies have devoted to the role of T cells in this disease [1, 4, 28-30]. Dubey *et al.* found elevated levels of interferon-gamma and tumor necrosis factor-alpha (TNF-α) in BM plasma of SAA patients [28], and these cytokines can induce apoptosis of CD34+ BM cells [31]. Hara *et al.* demonstrated excessive production of TNF-α by BM T cells and higher sensitivity of HSCs to TNF-α in patients with SAA [30]. However, up to 30% of patients have no immune-associated evidence and respond poorly to immunosuppressive therapy [1]. Pathogenesis of SAA remains to be Determined.

Primary HSC deficiency, including decrease in number and dysfunction, has also been proposed to account for the development of SAA [32-35]. *In vitro* long-term BM culture provided evidence for primary HSC dysfunction in the regenerative capacity and in the response to various cytokine stimuli [32, 33]. Abnormal telomere shortening of HSCs was found in some patients with SAA [34]. However allogeneic HSC transplantation cannot cure all patients, suggesting that other mechanisms exist.

Another important concept proposed in the context of SAA is related to the deficiency or dysfunction of BM microenvironment. MSCs are an essential component of the hematopoietic microenvironment which is appropriate for HSCs to survive, proliferate and differentiate [6, 7, 9-11]. MSCs provide an appropriate scaffold and a complex network of cytokines, adhesion molecules, and extracellular matrix proteins that are crucial for hematopoiesis [6-11]. Many studies have reported the promotive effect of MSCs for HSC expansion *in vitro* [7, 36-39]. In 2000, Koc *et al.* found rapid hematopoietic recovery after coinfusion of autologous MSCs at the time of HSC transplantation [40]. Accordingly, Lazarus *et al.* presented a multicenter trial of 46 patients receiving allogeneic HSCs and MSCs from HLA-identical siblings, and found prompt hematopoietic recovery in most patients, suggesting that the beneficial effect of MSCs on engraftment may relate to their supportive role in the hematopoiesis [41].

Only several studies have been conducted on the relationship between MSCs and SAA [12-14]. Bacigulupo *et al.* found that BM MSCs of SAA patients were deficient in their ability to suppress T cell proliferation and cytokine release, suggesting the lack of MSC immunoprotection in SAA BM [12]. Wu *et al.* investigated GATA gene expression of MSCs from chronic aplastic anemia patients, and

proposed that aberrant expression of these genes in BM MSCs may influence the BM microenvironment and lead to abnormal hematopoietic regulation [13]. In current study, we observed poor potential of proliferation and differentiation in BM MSCs derived from SAA children. The above studies provide strong evidence for MSC defects in SAA BM.

Several investigations have found that MSCs can not be acquired efficiently from umbilical cord blood of full-term infants, suggesting that MSCs are sparse or absent in cord blood [19-21]. Rubinstein *et al.* reported 562 recipients of cord blood HSC transplantation and found successful engraftment reduced significantly among SAA patients [42]. This can be explained, at least in part, by the insufficiency of BM hematopoietic microenvironment in SAA patients resulting from MSC defects and the few amount of MSCs provided by cord blood during transplantation. Therefore, cotransplant of MSCs and HSCs could be a potential strategy to treat SAA patients.

4.3 研究限制

Our study was of course limited by the small number of patients and the diseases of controls.

第五章 結論與建議

According to our results, BM MSCs derived from children with SAA had poor potential of proliferation and differentiation and these alterations may be important in the pathogenesis of the disease. Our findings should be useful in further understanding of the pathophysiology of SAA and consequently lead to the development of novel treatment modalities. Further studies are needed to elucidate the relationship between MSCs and SAA.

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Figure 1. MSCs of controls and SAA children shared a similar spindle-shaped morphology (A, ×100 magnification) and a consistent immunophenotypic profile which was negative for CD45, CD14 and CD34, and positive for CD105, CD73, and CD44 (B). Black and dotted lines indicate cultured cells from controls and SAA children stained with antibodies, respectively. The respective isotype controls are shown as grey lines.

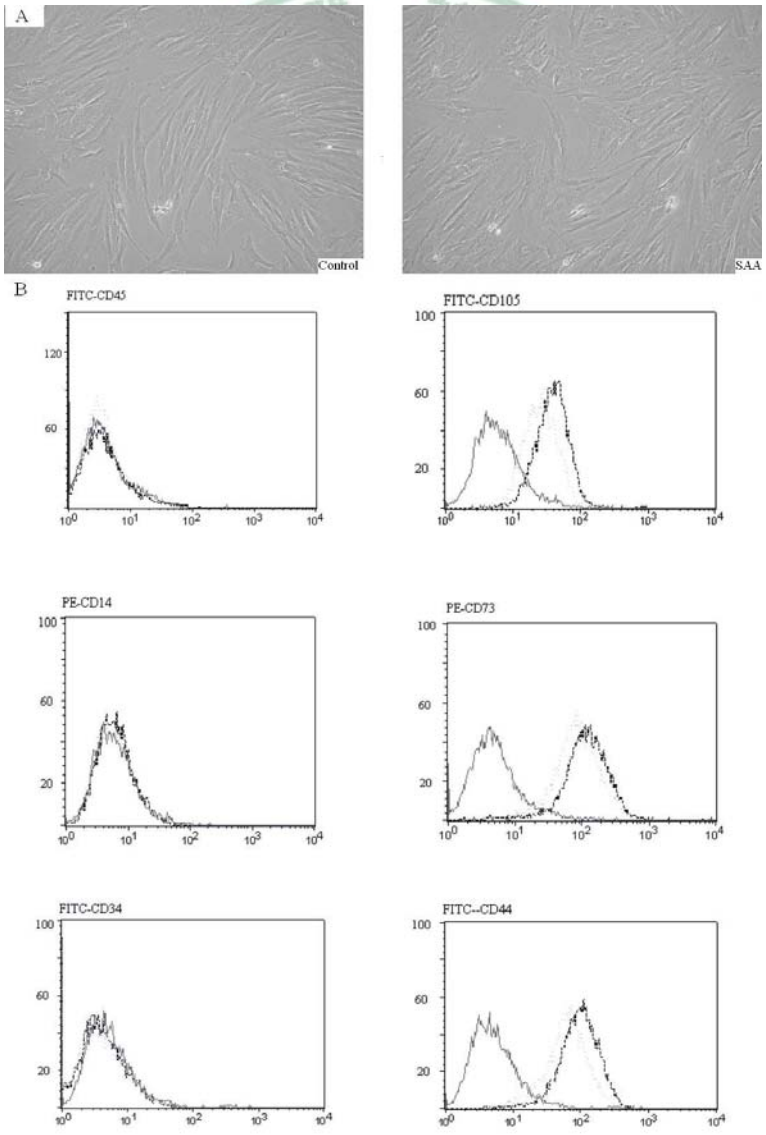

Figure 2. Proliferative capacity. (A) Average PD of passage 4 to 6 of control and SAA group. (B) Comparison of cumulative PD, shown as mean and 95% confidence interval. Each circle represents a subject studied.

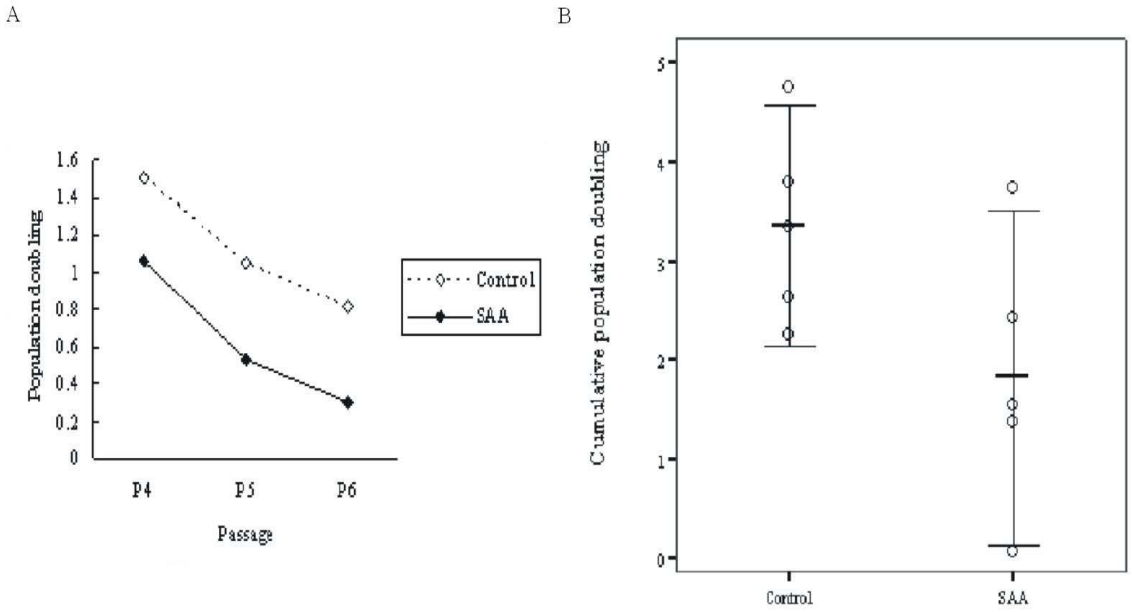

Figure 3. Differentiation potential. Osteogenic differentiation was demonstrated by ALP activity (A) and von Kossa stain (B) after 3-week induction. Adipogenic differentiation was demonstrated by Oil red O stain (C) after 2-week induction. Magnification of micrographs: ×100.

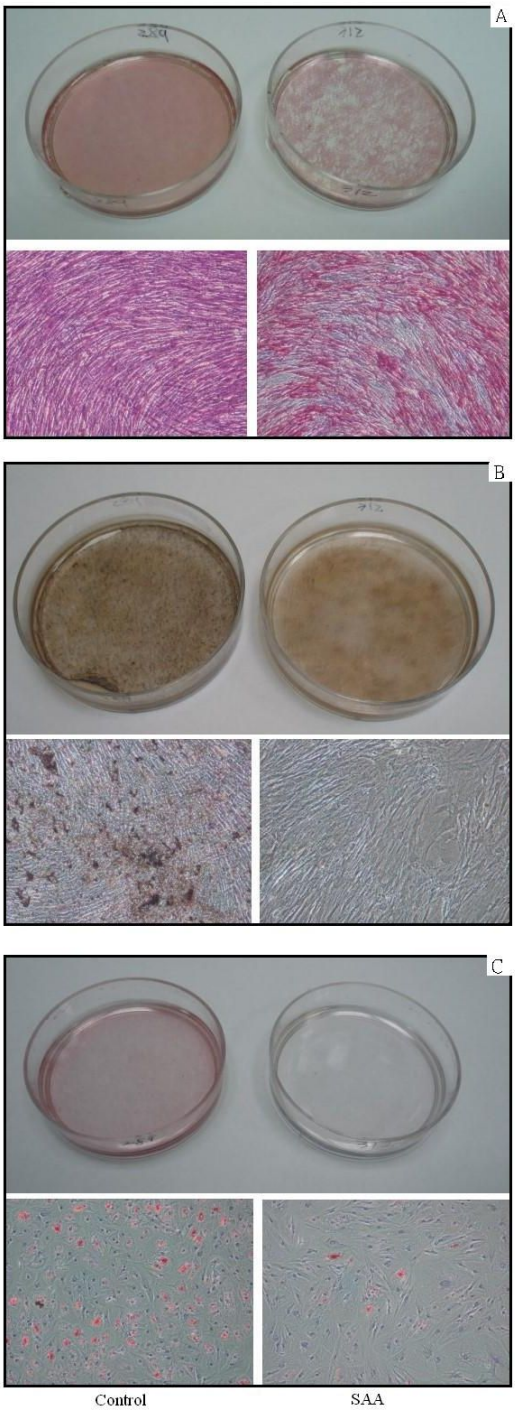

Figure 4. Comparison of osteogenic and adipogenic potential by quantification of ALP activity (A) and Oil red O (B) stain spectrophotometrically, respectively. Mean and 95% confidence interval are illustrated. Each circle represents a subject studied.

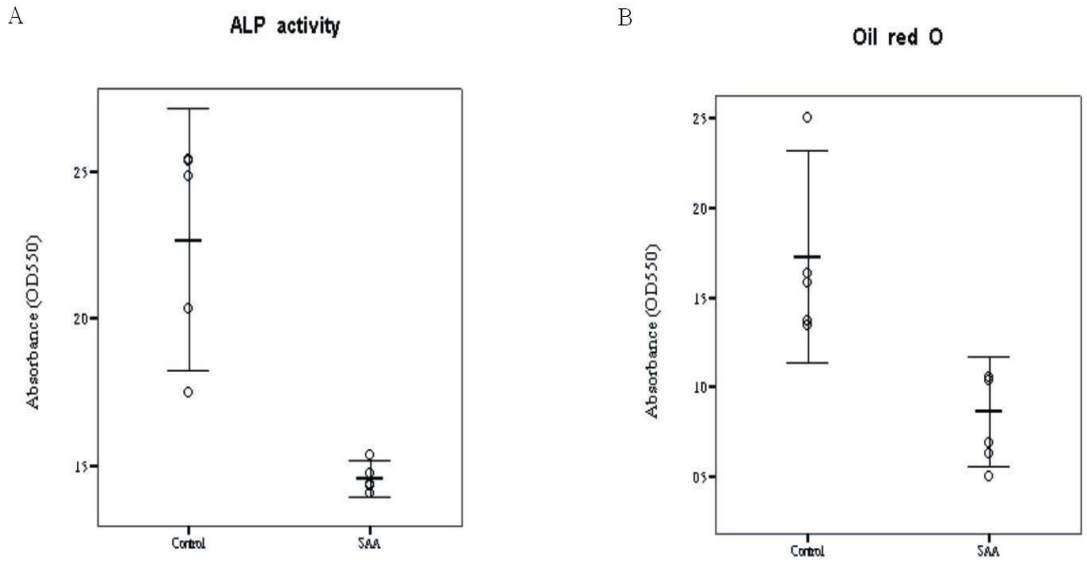

Figure 5. Comparison of differentiation potential by lineage-specific gene expression, Cbfa1 for osteogenesis (A) and lipoproteinlipase for adipogenesis (B). Mean and 95% confidence interval are illustrated. Each circle represents a subject studied.