The pluripotency and differentiation ability of human adipose-derived stem cell cultured on different ECM-coated surface

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Abstract

Human adipose derived stem cells (hADSCs) possess pluripotency and differentiation abilities. hADSCs are considered as an attractive prospect for regenerative medicine and tissue engineering. However, long term culturing of hADSCs was suffered from aging problem. It includes limitation passage numbers, pluripotency and differentiation ability was decreasing followed by passage numbers. Because microenvironment plays an important role in pluripotency gene expression and differentiation ability of hADSCs. In this study, I (Yu-Hsuan Chen) will investigate the differentiation ability and passage numbers of hADSCs culturing in different cell culture substrates.

Substrates in this study are prepared from the tissue culture polystyrene (TCPS) coating with different extracellular matrices (ECMs); (1) Matrigel, (2) Synthemax II (oligo-vitronectin based substrate), and (3) human recombinant vitronectin (rVN), where Matrigel is xeno-contained materials and the others are xeno-free materials.

The purpose of this project is (1) to find out the optimal environment for cultivation of hADSCs, (2) to characterize hADSCs detached from different ECM surface, and (3) to investigate the differentiation ability into osteoblasts of hADSCs detached from the dishes.

The interaction among hADSCs, substrates, and culture mediums affects pluripotent gene expression, which is investigated by osteogenic differentiation ability. We will determine the optimal ECM to keep their stemness for a long period and utilize hPL to build up a xeno-free culture condition for clinical applications in the future.

Motivation and Significance

hADSCs are considered to be excellent stem cells source for therapeutic application of regeneration of severe damaged tissues. Over the past few years, stem cells therapy has been extensively studied for clinical application for heart diseases and aesthetic medicine [1]. Among various stem cells, hADSCs have the potential to differentiate the mesenchymal, ectodermal and endodermal lineages and are easy to harvest. Additionally, adipose tissue is rich with stem cells and harvesting these cells is minimally invasive to donors, making it an ideal tissue to work with. Many pre-clinical animal studies have also demonstrated the promising therapeutic results by using hADSCs transplantaion. Furthermore, there have been several clinical trials showing the positive results in acute myocardial infarction by using hADSCs transplantaion [2].

Stem cells have the capacity to maintain prolonged self-renewal, as well as the ability to differentiate into specific cells lines. Given their unique regenerative abilities, hADSCs offer new potentials for use in clinical therapy and regenerative medicine. Optimizing cell growth for therapeutic use requires both rapid expansion of cells and cellular characterization including pluripotency and differentiation abilities that may be crucial to promote healing. For clinical usage of hADSCs, it is vital to examine the ability of a xeno-free system to support the cell expansion while maintaining the cellular capacity of hADSCs. In this context, considerable efforts are undertaken to grow human embryonic and adult stem cells under strict xeno-free culture conditions to eliminate or reduce the risk of adverse side effects due to xeno-contained materials used before.

According to the previous research, there are several evidences showing that hADSCs can be cultured under xeno-free environments and proliferate continuously. By using hPL as substitution of FBS to prepare the medium, both 5% and 10% hPL can enhance not only the proliferation speed but the pluripotency and osteogenic differentiation ability of hADSCs. The results also indicate that Synthemax II has the best performance on osteogenic differentiation of hADSCs among xeno-free ECMs. Though TCPS still possess the highest oteogenesis ability among all dishes, this previous study confirms the feasibility of xeno-free hADSCs cultivation conditions.

As all describe above, in this project, I will characterize hADSCs detached from different ECM surface and compare cell growth with the nutrient supplement FBS to which with hPL. Moreover, I will investigate the pluripotency and differentiation ability into obsteoblasts of hADSCs detached from the dishes. Finally, I will find out the optimal cultural conditions for cultivation of hADSCs.

Index of content

Chapter 1. Introduction

1.1 Adipose derived-stem cells (ADSCs)

ADSCs are isolated from the stromal vascular fraction (SVF), which is obtained from adipose tissue by enzymatic digestion. During the past few decades, ADSCs have been studied as an abundant, accessible, and replenishable source for tissue regeneration. These ADSC cells are multipotent, differentiating along the adipocyte, chondrocyte, myocyte, neuronal, and osteoblast lineages, and can also serve in other capacities, such as providing hematopoietic support and gene transfer [3].

In the 1960s, Rodbell firstly found the method to isolate cells from rat adipose tissue [4]. In the 2000s, Zuk et al. modified and developed ADSC isolation method from human adipose tissue and suggested that ADSC as a promising source for adult multipotent stem cells [5]. Recently, Nicoletti et al. also developed a new ADSC isolation protocol using enzymatic digestion [6]. D'Andrea et al. reported a successful long-term (four months) and large-scaled ADSC culture [7]. However, it is still necessary to establish a standardized ADSC isolation protocol, which can warrant homogenous ADSC population and reproducible results for clinical application. ADSCs have tendency to differentiate not only into adipocytes, but into other lineage cells including osteoblasts, chondrocytes, endothelial cells, and myogenic cells in vitro, which are comparable to bone marrow derived mesenchymal stem cells (BMSCs) [8] [9]. Similar to BMSCs, ADSCs secrete many different kinds of growth factors and cytokines, which could contribute in stimulating angiogenesis as well as myocardial regeneration [10].

1.2 Extracellular marix (ECM)

Both stem cell themselves and the microenvironment regulate cellular characteristics such as proper differentiation and maintenance of pluripotency. Thus, it is necessary to culture or expand the stem cells with biomimetic cell culture protein or ECMs where the cells are able to behave in their native tissue or the functional tissue that is needed for reconstructing or replacing the damaged or lost parts. Three ECMS mentioned below are utilized to conduct the project [11].

Matrigel is primarily composed of lamainin, collgen type IV, and enactin, which assume cells with the adhesive peptide sequence where the cells encounter in their native growth environment [12]. In addition, Matrigel is shown to be the optimal matrix for cell culture because it can promote the differentiation and proliferation of various cell types due to its growth factors. Matrigel is applied to proliferate human embryonic stem cells and mimic the ECM in cancer and stem cell culture [13].

Synthemax II is a novel animal-derived component-free (ACF) substrate and can self-adsorb onto tissue culture plastic or glass surfaces. This feature imparts versatility when scaling up production of therapeutic cells and facilitates a wider array of characterization assays. Synthemax II includes the RGD-containing sequence from the human vitronectin which promotes adhesion in various cells. Moreover, Synthemax II is less expensive than Synthemax R. To date, Synthemax II has been shown to support human iPSCs and human mesenchymal stem cells [14].

Vitronectin is a 75kDa glcorprotein, consisting of 459 amino acid residues. Vitronectin can be found in plasma, serum and tissues. Vitronectin promotes cell adhesion and expanding, inhibiting the cell membrane damage effect of the terminal cytolytic complement pathway, and binds to several serpin-serine protease inhibitors. Vitronectin and fibronectin are one of the major cell adhesion proteins in plasma.

Chapter 2. Materials and Methods

2.1 Preparation of ECM-coated dishes for cell culture

Three different ECMs (Matrigel, Synthemax II, human recombinant vitronectin) for coating dish are prepared by formulas showed in **Table 1**. The ECMs are coated onto TCPS surfaces (12-well plates, surface area = 3.71cm^2) for two hours in the room temperature. Before seeding hADSCs, the dishes are rinsed twice by PBS.

Extracellular matrix (ECM)	**Basis**	**Stock concentration**	**Coating concentration**	**Stock volume**	**Solvent (PBS) volume**
Matrigel	Mixture	0.1 mg/mL	0.1 mg/mL	-	-
Synthemax® II	Oligo-vitronectin	-	25 μg/ml	2.5 μL	97.5μL
rh-Vitronectin	Vitronectin	500 μg/mL	5 μg/mL	1 μL	99 μL

Table1. The formula of ECM-coated dishes.

2.2 Isolation of human adipose tissue

Human adipose tissue is cut and immersed in phosphate buffer saline (PBS) containing 1% of antibiotic-antimycotic to remove blood and impurities. After the washing process, the adipose tissue is minced into tiny fragments and digested with equal volume digestion solution (0.75 mg/mL of collagenase, 20 mg/mL of BSA), which is vigorous shaken in water bath at 37℃ for two hours. After the digestion is completed, the digestion mixture solution is passed through a 100 μm filter to remove large and undigested tissue pieces and centrifuged at 1800 rpm for 7 min to separate the floating part from stromal vascular fraction (SVF). After centrifugation, the supernatant is carefully discarded without disturbing the cell pellets by pipetting. The cells are re-suspended with ACK lysing buffer for breaking red blood cells and put in the CO_2 incubator for 5 min at 37℃. Finally, the cell-ACK is centrifuged at 2000 rpm for 7 min and the primary cell solution is seeded on desired substrate with proper culture medium.

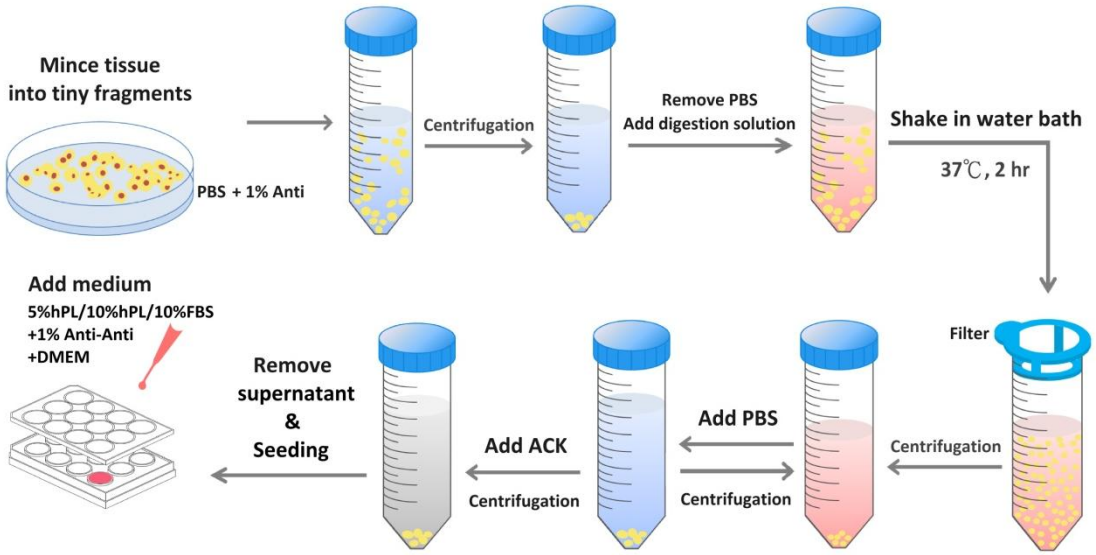


Figure1. Isolation procedure of human adipose tissue.

2.3 hADSC cultivation

hADSCs are cultured for seven days for cell expansion to be confluent as one passage. In a passage, hADSCs are seeded on plates coated with three different ECMs mentioned above and on TCPS as the control condition. hADSCs are cultivated on the above cell culture dishes at the same conditions (seeding density: 6000 cell/cm^2 in 5% CO_2 incubator with stable humidity at 37℃). The cell culture media used for hADSC cultivation are media containing 10% FBS. There is the serum formula for medium in this study, 4.5 mL of FBS and 450 μL of antibiotic-antimycotic are added into 40 mL of basal medium. Both the basal and culture medium are stored at 4℃ and avoided light. The medium is exchanged with fresh medium for every two days. Expansion of hADSCs on the dishes is investigated by counting the cell density under optical microscope.

2.4 Characteristic evaluation of hADSCs

This part of the characterization is the osteogenic differentiation ability of hADSCs. hADSCs are cultured in osteogenic culture medium for 28 days. The culture medium is exchanged to be fresh medium once every two days. At the day 14th of differentiation, the alkali phosphatase (ALP) activity is evaluated using ALP detection kit by following the manufacture's protocol. The gene expression of Runx2 is evaluated using qRT-PCR assay to check whether the hADSCs differentiation into early stage of osteoblasts. At the day 28th, the Alizarin Red S staining and von Kossa staining of hADSCs are performed using standard procedures. The gene expression of Osteriox is also evaluated. This step aims to evaluate the degree of the late stage differentiation of hADSCs into osteoblasts.

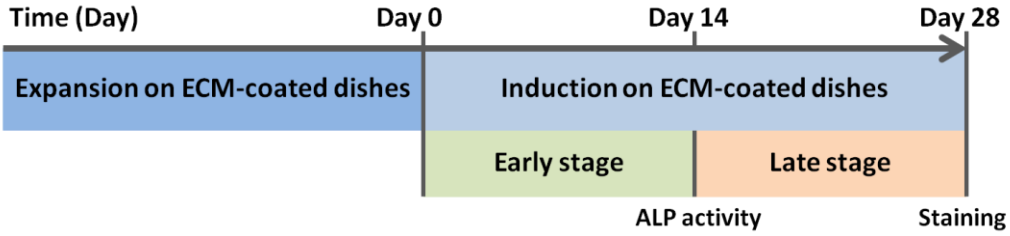
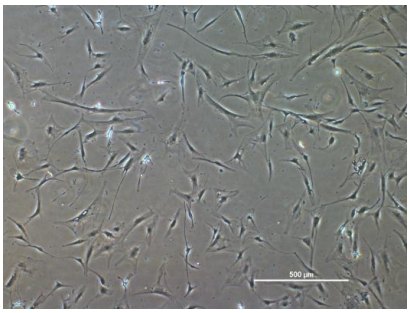
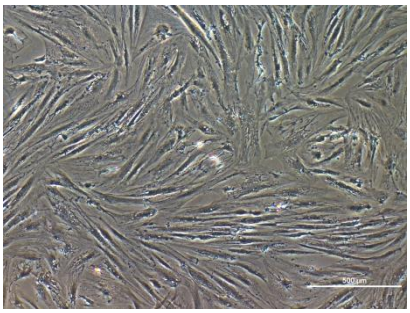
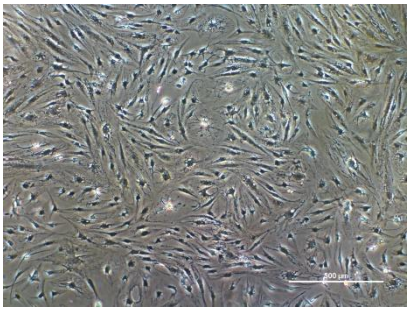
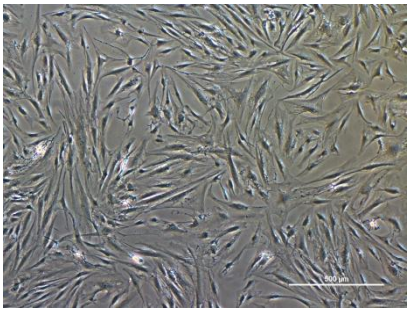


Figure2. The time line of osteogenic differentiation of hADSCs.

Chapter 3. Present Results

3.1 Cultivation of hADSCs

ADSCs were cultivated on TCPS by using the density 6000 cells/cm^2 and medium containing 4.5 mL of FBS, 450 μL of antibiotic-antimycotic, and 40 mL of basal medium and recorded the morphology and the amount of cells during passage. The morphology of cells cultured on TCPS from passage 3 to passage 6 is shown in **Figure 3**; and **Figure 4** shows the doubling time and standard bar of hADSCs cultured on TCPS from passage 3 to passage 7.

	Day 2	Day 7
Passage 3		
Passage 4		
Passage 5		

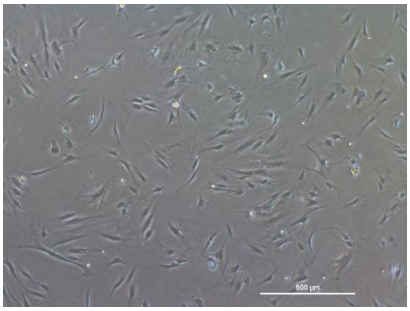
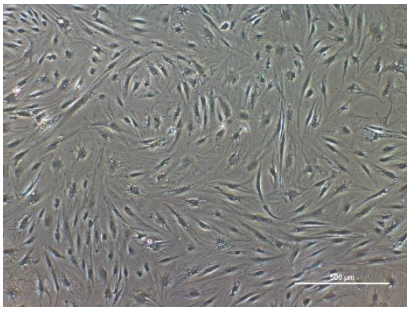
Passage 6		

Figure 3. Morphology of hADSCs on TCPS from passage 3 to passage 6 on day 2 and day 7**.** Scale bar = 500µm.

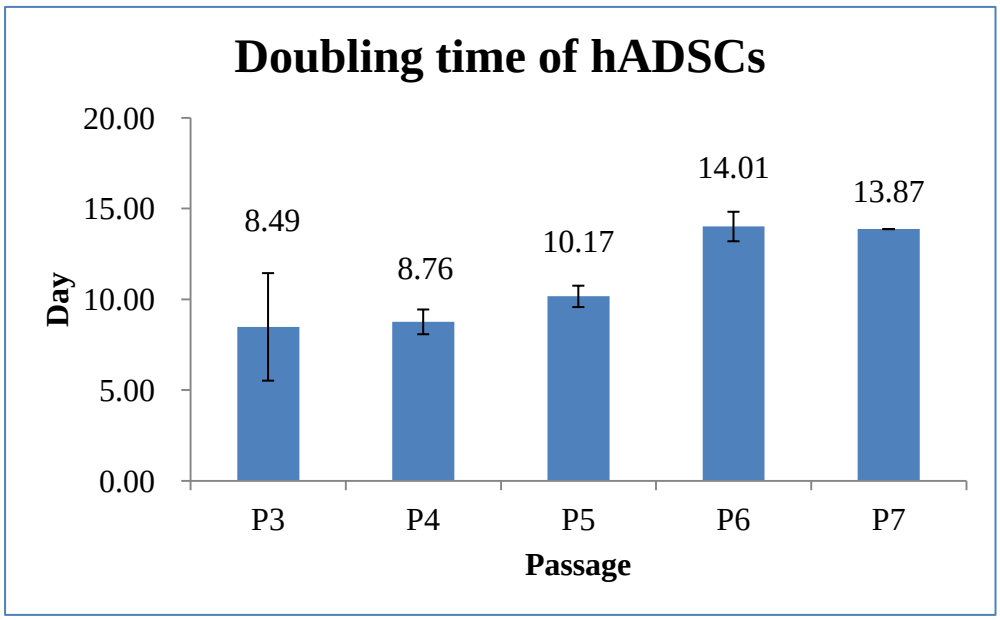


Figure 4. The doubling time of hADSCs on TCPS from passage 3 to passage 6.

ADSCs were also cultivated on Matrigel by using the density 6000 cells/cm^2 and medium containing 4.5 mL of FBS, 450 μL of antibiotic-antimycotic, and 40 mL of basal medium and recorded the morphology. The morphology of cells at passage 3 cultured on TCPS and Matrigel-coating dishes are shown in **Figure 5**.

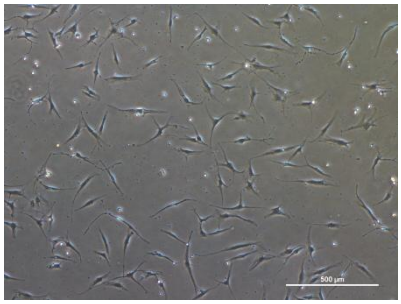
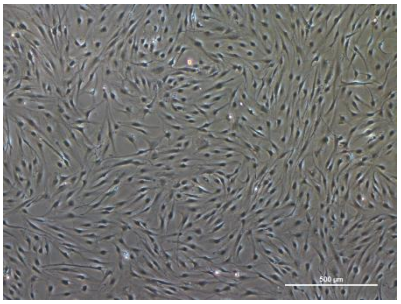
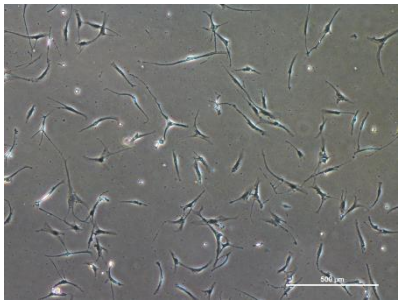
	Day 2	Day 8
TCPS		
Matrigel		

Figure 5. Morphology of hADSCs on TCPS and Matrigel-coating dishes at passage 3 on day 2 and day 8**.** Scale bar = 500μm.

3.2 Differentiation abilities of hADSCs

ADSCs were pre-cultured on TCPS and Matrigel-coating dishes by using the density 15000 cells/cm^2 until 90% confluence and induced into osteoblasts by osteogenic differential medium. The differential medium is home-made medium containing 4.5 mL of FBS, 450 μL of antibiotic-antimycotic, 40 mL of basal medium, 10nM dexamethasone, 20mM β-glycerophosphate, and 50uM L-ascorbric acid 2-phosphate. The morphology of cells at passage 4 during osteogenic differentiation on TCPS and Matrigel-coating dishes are shown in **Figure 6**.

	Day 14	Day 28
TCPS		
Matrigel		

Figure 6. Morphology of oseoblasts-induced cells on TCPS and Matrigel-coating dishes at passage 4 on day 14 and day 28**.** Scale bar = 500μm.

3.2.1 Alkaline Phosphatase (ALP) test

After induction of hADSCs into osteoblasts for 14 days, the differentiation ability of cells was analyzed by measuring ALP activity, the early stage marker of osteogenesis. Sensolyte pNPP Alkaline Phosphatase Assay Kit was used in the analysis process. ALP standard curve was set up by measuring the absorption of diluted alkaline phosphatase standard solution and is shown in **Figure 7**. ALP activity of differentiated cells from hADSCs cultured on TCPS and Matrigel-coating dishes is shown in **Figure 8**. The cells cultured on TCPS showed higher expression of ALP activity compared to those on Matrigel-coating dishes.

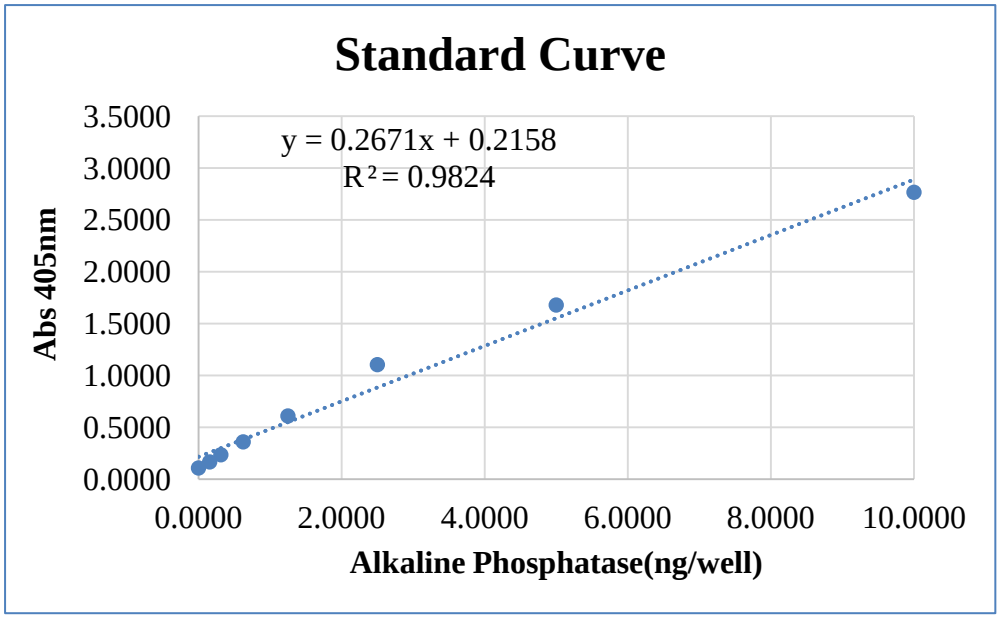


Figure 7. ALP standard curve

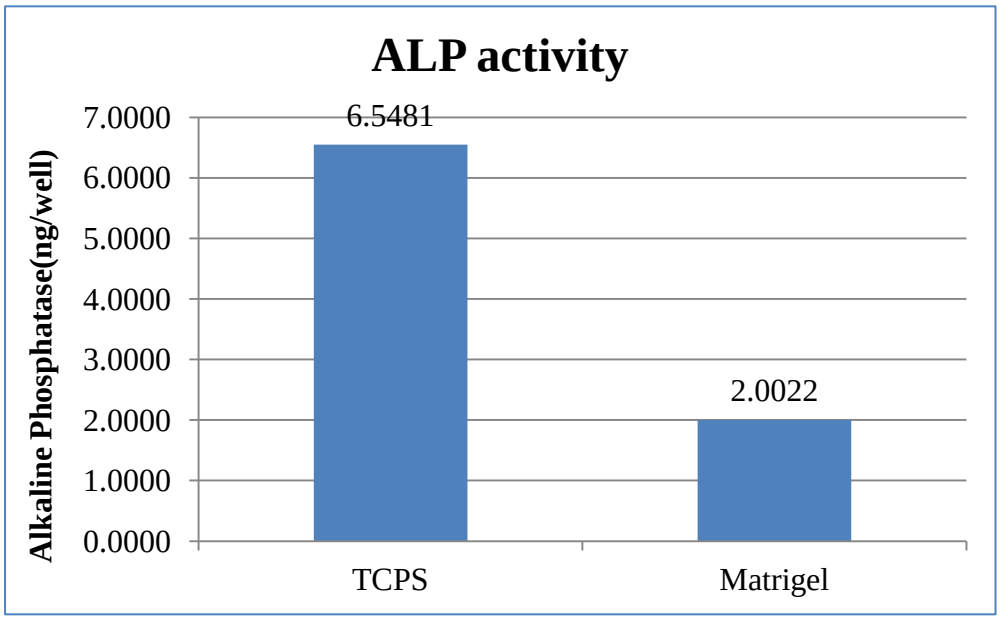


Figure 8. ALP activity (early stage marker of osteoblasts) of ADSCs on TCPS and Matrigel-coating dishes after 14 days induction into osteoblasts.

3.2.2 Alizarin red S staining and von Kossa staining

After induction of hADSCs into osteoblasts for 28 days, Alizarin red S and von Kossa staining staining were conducted to measure the level of the mineralization. Cells cultured on TCPS showed higher level of osteogenic diffrentiation from Alizarin red S and von Kossa staining (late stage marker) where the staining ratio from the staining picture was quantified by Image J software (**Fig. 9** and **10**, respectively).

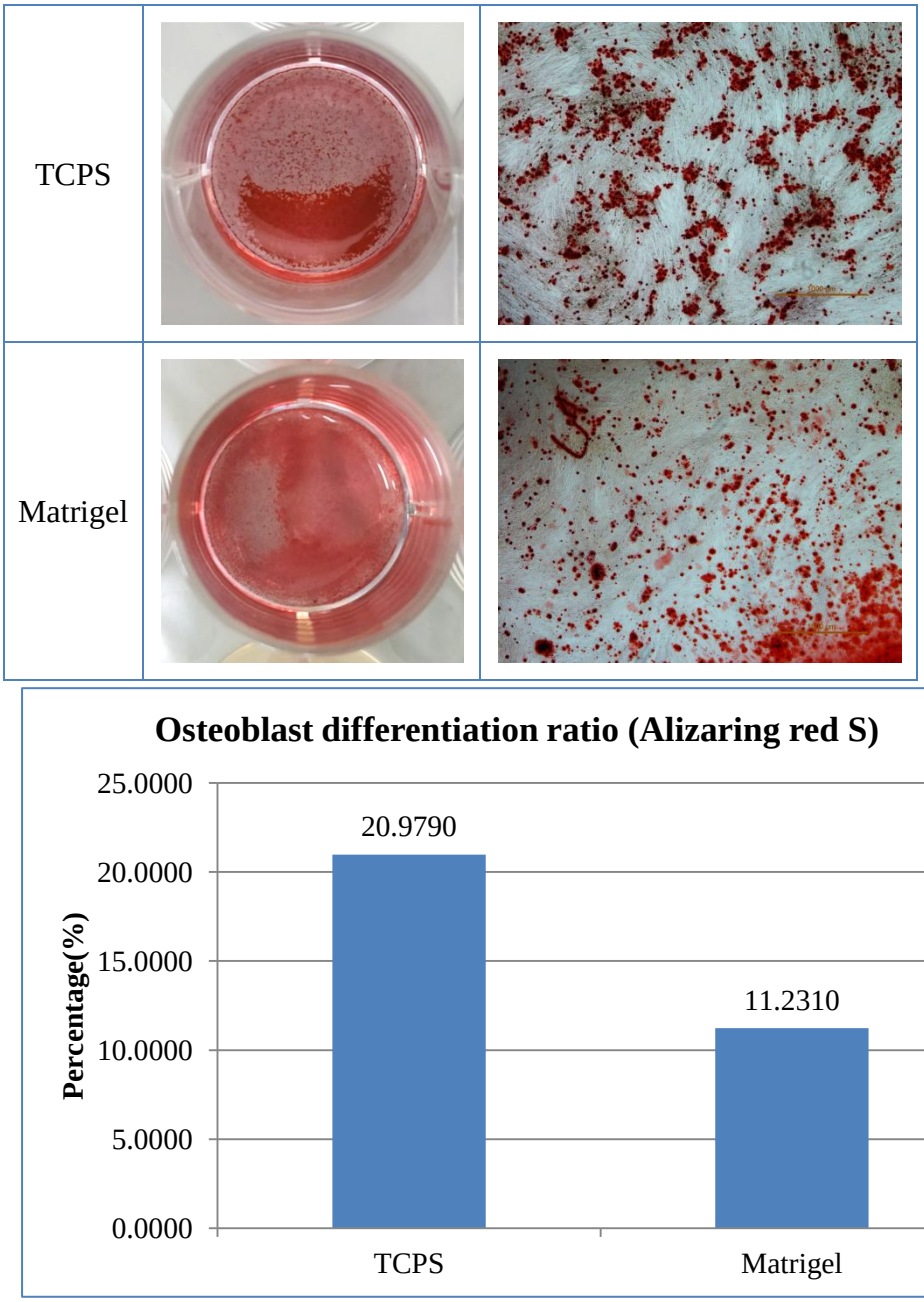
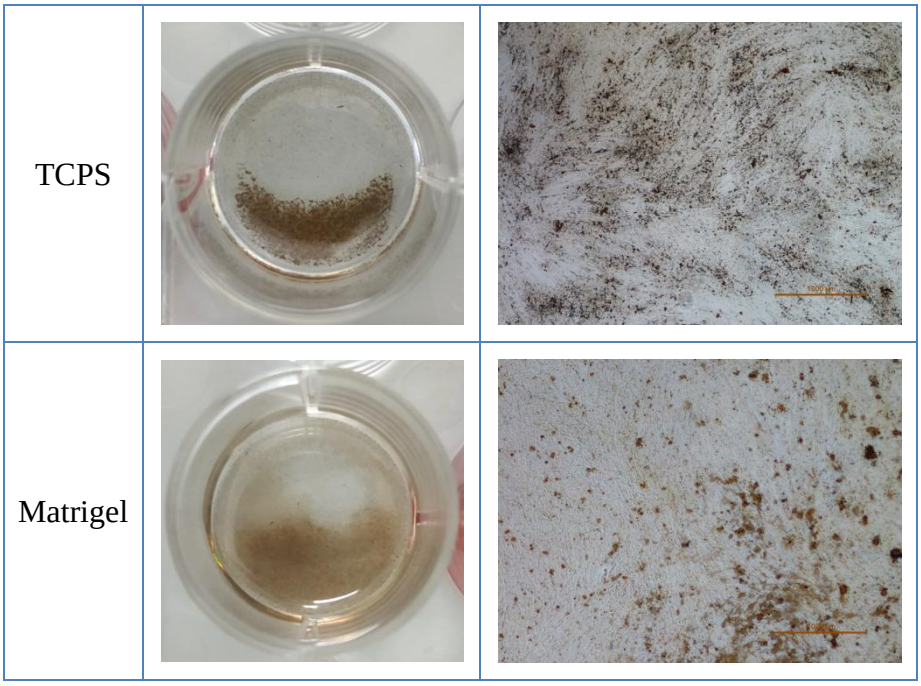


Figure 9. Osteogenic differentiation of hADSCs on TCPS and Matrigel-coating dishes after 28 days induction into osteoblasts. (A) Top view of the cells stained with Alizarin Red S. (B) Morphology of the cells stained with Alizarin Red S under microscopy, (C) the quantification of staining cell ratio of Alizarin Red S. Red color sites indicate calcium deposition. Scale bar = 1000μm.



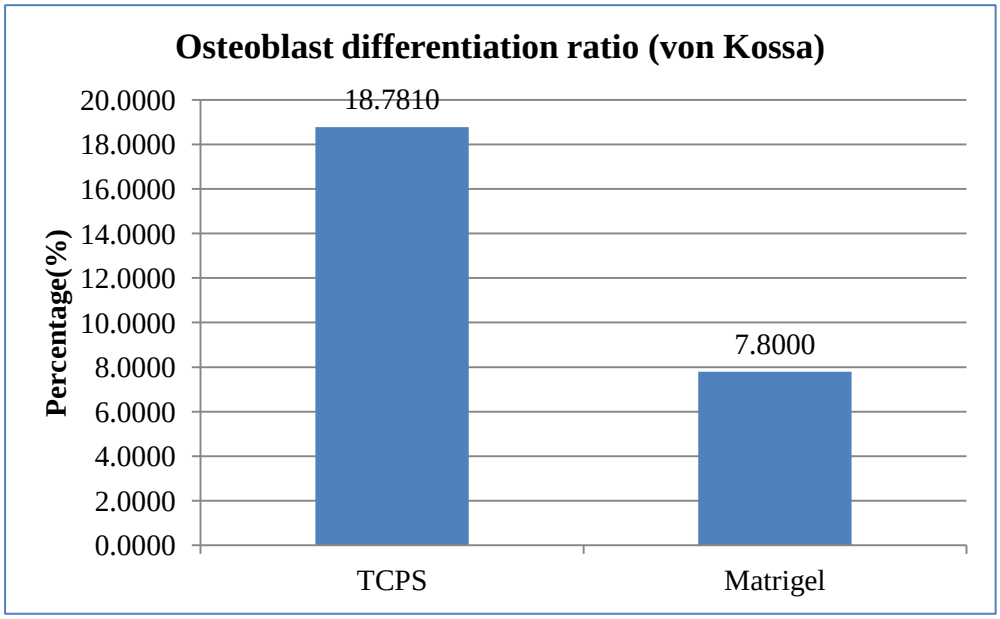


Figure 10. Osteogenic differentiation of hADSCs on TCPS and Matrigel-coating dishes after 28 days induction into osteoblasts. (A) Top view of the cells stained with von Kossa. (B) Morphology of the cells stained with von Kossa under microscopy, (C) the quantification of staining cell ratio of von Kossa. Black color sites indicate calcium phosphate deposition. Scale bar = 1000μm.

3.2.3 Isolation of total RNA

Novel Total RNA Mini Kit was used during the isolation, and Silica column can adsorb RNA via high ionic strength. The concentration of RNA was measured by spectrophotometer, and that of induced cells on TCPS at day 14 is 35.3 μg/mL and that of induced cells on Matrigel-coating dishes is 144.8 μg/mL. The isolated RNA was stored at -80℃ refrigerator, and the data and RNA are needed in the Quantitative real time polymerase chain reaction (qRT-PCR) for genetic test of osteoblast.

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