Differentiation of Human Adipose-Derived Stem Cells into Parathyroid Hormone-Secreting Cells

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Research Article

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Abstract

Introduction

Complications arising from neck surgery are the most common causes of acquired hypoparathyroidism. Parathyroid hormone (PTH) regulates serum calcium and vitamin D levels. The current treatments for hypoparathyroidism, including PTH supplements and high doses of calcium and vitamin D, are a life-long burden and may cause side effects. Recent studies have shown that parathyroid gland differentiation occurs from embryonic stem cells, thymic epithelial cells, and tonsil-derived stem cells. Because adipose-derived stem cells (ADSCs) do not face ethical concerns raised for studies with human embryonic stem cells, in the present study, we attempted to induce differentiation of ADSCs into parathyroid cells.

Methods

ADSCs were isolated from abdominal subcutaneous adipose tissues obtained by plastic surgery from three male donors (mean age, 40 years). For differentiation into parathyroid-like cells, ADSCs were incubated in minimum essential medium-alpha supplemented with activin A and soluble Sonic hedgehog for 7–21 d. The parathyroid differentiation markers *PTH*, glial cells missing homolog 2 (*GCM2*), and chemokine (C-C motif) ligand 21 (*CCL21*) were detected using real-time quantitative polymerase chain reaction to confirm differentiation.

Results

The ADSCs exhibited flat and vacuolated morphology, which changed into secretory parathyroid gland–like nodules on day 21 after differentiation. The mRNA expression of *PTH, GCM2,* and *CCL21* increased in the differentiated cells. Furthermore, a significant amount of PTH protein was detected in differentiated cells on day 7 post-differentiation.

Conclusion

Human ADSCs isolated from adipose tissues successfully differentiated into PTH-secreting cells. ADSC-secreted PTH may be a promising therapeutic for hypoparathyroidism patients.

1. Introduction

The most common causes of acquired hypoparathyroidism involve complications arising from neck surgery. Hypocalcemia, induced by hypoparathyroidism, is characterized by various clinical symptoms ranging from numbness and tingling of fingers or toes to life-threatening bronchospasm, seizure, and congestive heart failure [1]. The incidence of permanent hypoparathyroidism ranges from 20–25% [2,3]. Currently, autograft is the best procedure to prevent post-surgical hypoparathyroidism, with a success rate of 50–95%. However, there is no opportunity to perform an autograft if parathyroid damage is not detected during surgery [4].

Parathyroid hormone (PTH) regulates serum calcium and vitamin D levels through its action on bones, kidneys, and gastrointestinal tract. Theoretically, supplementation of PTH is the best treatment for hypoparathyroidism; administration of full-sequence PTH (1–84) for 24 months reduces the requirement for calcium and 1,25-dihydroxyvitamin D [5]. In 2015, Natpara (PTH 1–84) was approved by the Food and Drug Administration for hypoparathyroidism treatment. While PTH has a short half-life of 4 min and PTH secretion exhibits diurnal variation, injection of PTH (1–34) once or twice daily shows considerable efficacy in decreasing fluctuation in serum calcium levels and excretion of urine calcium [6,7]. However, its high cost and need for daily injections remain a lifelong burden. Therefore, the commonly used treatment for hypoparathyroidism is supplementation with high doses of calcium and vitamin D; however, hypercalciuria, renal impairment, vitamin D intoxication, and ectopic calcification may be potential side effects [8]. Hence, researchers continue to explore alternative treatments for hypoparathyroidism.

The parathyroid is a relatively simple gland as each cell performs the organ function; normal function of the gland is possible even with a few cells and no structural arrangement of cells is required for its function [9]. Recent studies have achieved parathyroid gland differentiation from embryonic stem cells, thymic epithelial cells, and tonsil-derived stem cells [10–12]. Unlike human embryonic stem cells, adipose-derived stem cells (ADSCs) are relatively free from ethical concerns and can be obtained less invasively than other cell lineages. Therefore, to the extent of our knowledge, this study describes a method to differentiate parathyroid glands from human ADSCs for the first time.

2. Methods

2.1 ADSC donors and donor medical history

ADSCs were isolated from subcutaneous abdominal adipose tissues that were obtained from three male donors (mean age, 40 years) during plastic surgery. They exhibited normal serum calcium, phosphorus, and PTH levels. They had received no medications that influenced calcium levels, including calcium and vitamin D supplements, and anti-resorptive agents. Informed written consent was obtained from all participants in this study, and the study protocol was approved by the Institutional Review Board of the Pusan National University Hospital (IRB number 2013-8). All research was performed in accordance with the guidelines.

2.2 Differentiation of human ADSCs into parathyroid-like cells

Knife biopsies of adipose tissue were immediately put in minimum essential medium-alpha (MEM α, Gibco, Life Technologies Corp., Grand Island, NY, USA) which was containing 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Samples were transported to the laboratory and processed in less than 30 min outside body. Under aseptic condition, the tissues were chopped and digested with 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37 °C with vigorous shaking. Then, 25 mL MEM α supplemented with 10% fetal bovine serum (FBS) was added to neutralize the collagenase, and then centrifuged at 3000 rpm (1614 ×g) for 10 min. Digested tissue was subjected to filtration through a

70-μm nylon cell strainer (BD Biosciences, San Diego, CA, USA), after which the cells were washed with phosphate-buffered saline (PBS), and centrifuged again at 1600 rpm (459 ×g) for 10 min. The ADSCs obtained from sediment were expanded in ADSC culture medium of MEM α containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2. The medium was changed twice per week.

For parathyroid-like cell differentiation, ADSCs were incubated in MEM α supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 100 ng/mL activin A (PeproTech, Inc., TX, USA), and 100 ng/mL soluble Sonic hedgehog (Shh, PeproTech) for 7–21 d. The medium was changed every 4–5 d.

2.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Each sample containing 2 μg of RNA was heated at 65 °C for 5 min before addition of reverse transcriptase. cDNA was prepared through incubation at 42 °C for 60 min using the DiaStar RT kit (SolGent, Seoul, Korea), and RT-qPCR was performed on an ABI 7500 Real-time PCR system (Applied Biosystems) using a AccuPower® 2X GreenStar^{TM} qPCR Master Mix (Bioneer Corporation, Daejeon, Korea). PCR was set for 40 cycles, each consisting of pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s. The expression of the target gene was normalized to that of β-actin. The expression levels of genes specific to parathyroid-like cells were measured, such as *PTH*, glial cells missing homolog 2 (*GCM2*), and chemokine (C-C motif) ligand 21 (*CCL21*). The primer sequences were as follows: *PTH*, 5'-GAT CTC TTC CTG GGA AGA AG-3' (forward) and 5'-GAT ACC TGC AAA AGA CAT GG-3' (reverse); *CCL21*, 5'-GCC TCA AGT ACA GCC AAA-3' (forward) and 5'-GGG CAA GAA CAG GAT AGC-3' (reverse); *GCM2*, 5'-GCA ACA TAC CTC CCT TGG AA-3' (forward) and 5'-TGC CAA TTC ATA GCT GCA AG-3' (reverse); β-actin, 5'-AAC ACC CCA GCC ATG TAC G-3' (forward) and 5'-ATG TCA CGC ACG ATT TCC C-3' (reverse).

2.4 Western blot analysis

Cell proteins were isolated using PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea). Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). PTH protein was separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene fluoride membrane (Amersham Biosciences, Little Chalfont, UK). The membrane was blocked using 5% skim milk and incubated with PTH antibody (1:1,000 dilution; Abcam, Cambridge, UK) and GAPDH antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4 °C overnight each and subsequently with horseradish peroxidase–linked secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were detected using the ECL Western Blotting detection system (AB Frontier, Seoul, Korea).

2.5 Transmission Electron Microscopy (TEM)

The ADSC was fixed with 2.5% glutaraldehyde (4 °C, PBS, pH 7.2) for 24 h and then, was fixed with 1% osmium tetroxide in the PBS for 1 h at room temperature. The sample was dehydrated with a serial graded ethyl alcohol and embedded in epoxy resin (Epon 812 mixture). Thick sections (1 μm) were stained with 1% toluidine blue for light microscopy. Thin sections (50~60 nm) were prepared using an ultramicrotome (EM UC7, Leica Microsystems, Wetzlar, Germany) and were double stained with uranyl acetate and lead citrate. Thin sections were examined with a transmission electron microscope (JEM-1200 EXII, JEOL Ltd., Tokyo, Japan). We referred to the previous research method[13].

3. Results

Alterations in cell morphology were observed during parathyroid-like cell differentiation from ADSCs. As observed under light microscopy, flat and vacuolated ADSCs differentiated into secretory parathyroid gland-like nodules (Fig. 1). Transmission electron micrographs showed that the ultrastructure of ADSCs was also altered after differentiation. Compared with the control, distinct dense spots were observed in the lumen of differentiated ADSCs and this observation is typical in many secretory protein-releasing cells such as parathyroid cells (Fig. 2). RT-qPCR analysis revealed a considerablyincrease in mRNA expression of the parathyroid markers *PTH*, *GCM2*, and *CCL21* over time. Compared with the β-actin housekeeping gene, *PTH*, *GCM-2*, and *CCL21* were upregulated 13.59-fold, 8.10-fold, and 3.43-fold, respectively, on the seventh day of differentiation and 33.69-fold, 24.42-fold, and 6.09-fold, respectively, on the 21st day of differentiation ($p < 0.05$) (Fig. 3). Western blot analysis also revealed a significant amount of PTH protein on day 7, which increased over time (Fig. 4).

4. Discussion

In this study, we differentiated ADSC to parathyroid hormone–secreting cells. These cells have proven to be parathyroid gland cells by their expression of parathyroid cell markers, such as *PTH*, *GCM-2*, and *CCL21*, and by their secretion of PTH proteins.

Conventional therapy for hypoparathyroidism includes high doses of calcium and vitamin D. Due to low PTH, this may result in nephrolithiasis by hypercalciuria and extraskeletal calcification (kidney, lens, and basal ganglia) by calcium phosphate products [1,8]. PTH has anti-calciuric effects that induce renal calcium resorption in the distal convoluted tubule and anti-phosphorus resorption effects that inhibit renal phosphorus resorption in both the proximal and distal convoluted tubules [14]. Thiazide diuretics may help calcium resorption in the distal convoluted tubule, and are thus used to prevent hypercalciuria. Therefore, PTH replacement is a risk-free alternative to the side effects of conventional treatment of hypoparathyroidism.

As the N-terminal is the bioactive fragment of PTH, there are two type of recombinant parathyroid hormones: PTH_{1-84} and PTH_{1-34}. As the polypeptide is broken down when administrated orally, recombinant PTH needs to be injected subcutaneously [15]. Injection of recombinant PTH once a day causes fluctuation of calcium levels, which results in hypercalcemia in the first 6–8 h following injection.

Twice a day injection causes less fluctuation, but stimulates an above normal bone turnover rate and decreases bone mineral density, as PTH may increase cortical porosity [16]. Continuous PTH infusion with a pump results in less fluctuation of serum calcium and moderate stimulation of bone turnover [17]. However, continuous PTH infusion also causes inconvenience in a patient's daily life and needs regular blood sampling for estimation of serum calcium titers. Furthermore, there is a lack of data for estimating the optimal dose of PTH in children with autosomal dominant or autoimmune hypoparathyroidism.

Another solution to prevent acquired hypoparathyroidism during neck surgery is autografting. PTH produced by grafted parathyroid tissue allows patients to be free from painful daily injections and regular blood sampling for serum calcium titer as the grafted parathyroid tissue is a calcium sensing receptor itself. It takes 6–10 weeks for parathyroid function to normalize after an autograft. However, there is no opportunity to perform an autograft if gland damage is not detected during surgery.

Stem cell and tissue engineering is one of the ultimate fields in biomedicine. Human embryonic stem cells differentiate relatively easily compared with other cells, but its use sparks ethical controversies. Conversely, postnatal adult stem cells have no ethical implications and exhibit restricted differentiation potential. Adipocytes are differentiated from mesenchymal cells, and thus, adult mesenchymal stem cells can be derived from adipose tissues. ADSCs are multipotent with chondrogenic, neurogenic, and osteogenic abilities [18–20]. ADSCs may be differentiated into parathyroid cells using the Bingham protocol with Sonic hedgehog (Shh) [9]. In the study by Zhang et al. (2020), which demonstrates differentiation of rat ADSCs into parathyroid-like cells, it was seen that the higher the concentration of activin A, the greater the differentiation into parathyroid-like cells [21]. The differentiation method used in the present study involved 100 ng/mL activin A. To verify that the differentiated cells are parathyroid cells, many studies detect the levels of parathyroid gene expression markers *CCL21*, *GCM2*, *PTH*, and calcium sensing receptors using RT-qPCR [10,11,22]; PTH protein levels have also been detected using enzyme-linked immunosorbent assays [9,11,12]. Notably, *CCL21* and *GCM2* [23] are markers of parathyroid precursor cells.

The differentiation time of thymic stromal cells into parathyroid-like cells is long, i.e., approximately 10 weeks [10], and may cause problems in tissue preservation. In contrast, tonsil-derived mesenchymal stem cells differentiate maximally within 7 d [12], which is a relatively short time for differentiation; however, tonsil tissue cannot be obtained from inflammatory organs in limited cases such as chronic tonsillitis. In this aspect, ADSCs are technically more useful and show rapid differentiation within 7 d. Abundant and intact adipose tissue can be obtained effectively from all parts of subcutaneous as well as mesenchymal fat tissue by liposuction [24]. In case of liposuction, the procedure for harvesting adipose tissue is easy and safe, and involves fine mincing of tissues, with the stromal fraction enriched for ADSCs. Furthermore, even if the differentiation environment is not supplied, ADSCs survive adverse conditions of low glucose, glutamine, and oxygen concentrations [25]. Consequently, ADSCs may be used for native PTH production for the treatment of osteoporosis. Previous reports have shown that recombinant PTH is less active than the native protein [26]. Furthermore, PTH produced by differentiated tonsil-derived mesenchymal stem cells has a hundred times higher osteogenic capacity than that of recombinant human PTH [12]. In conclusion,

ADSCs are clinically useful as they exhibit rapid differentiation and can be obtained less invasively through methods like liposuction. ADSCs may also be used for native PTH production.

Declarations

Disclosure Summary: All authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

EH Kim searched the literature databases and co-wrote the original draft. SS Kim conducted conceptualization, investigation, methodology, data curation, formal analysis, project administration, supervision, validation, review, and editing, and co-wrote the original draft. JI Kim, JM Cheon conducted investigation and data curation. JH Kim, JC LEE, and KU Choi conducted supervision. IJ Kim conducted conceptualization and supervision. SG Wang conducted conceptualization, supervision, and validation. All authors interpreted the results, reviewed and approved the final manuscript.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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Figures

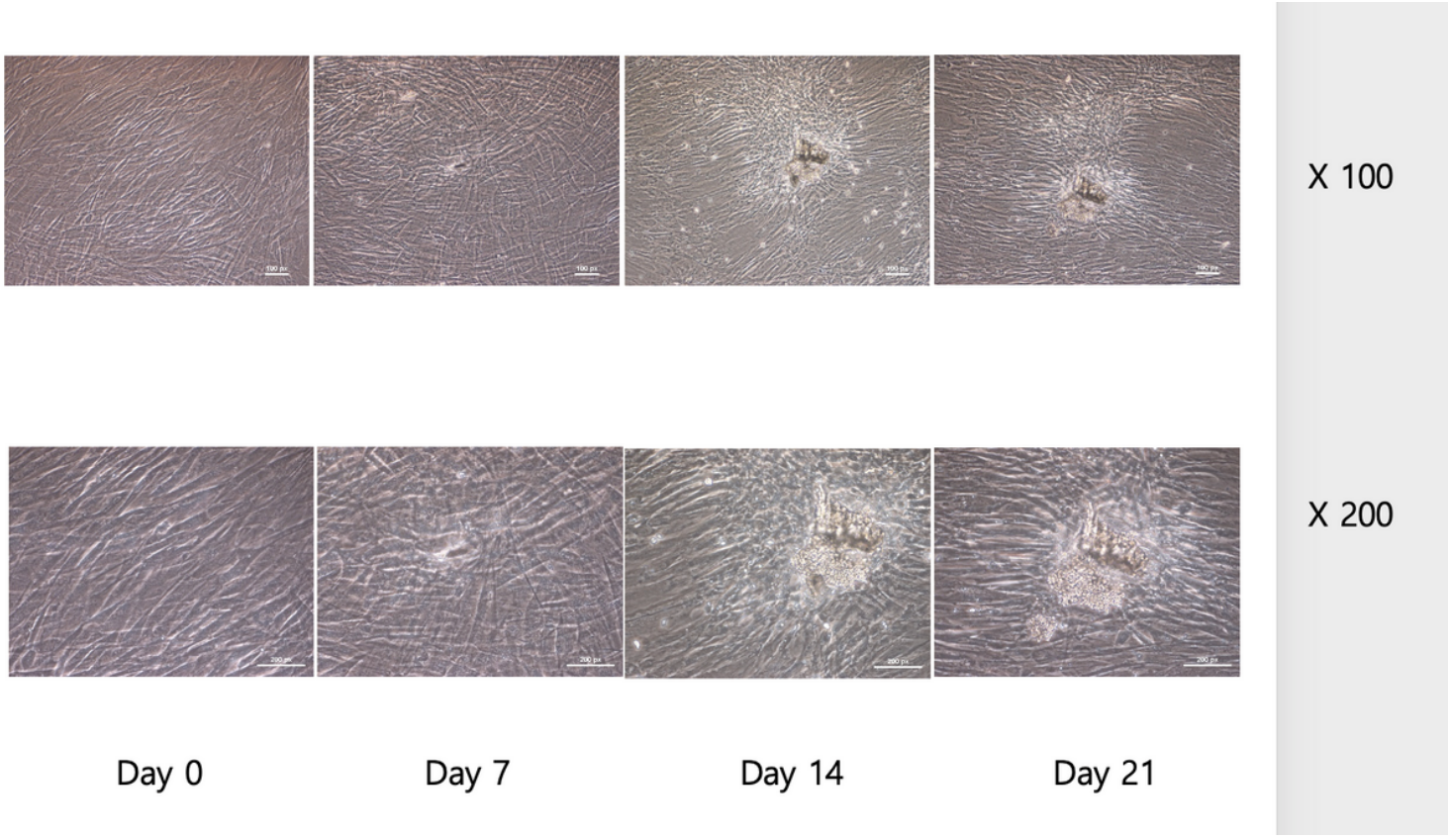
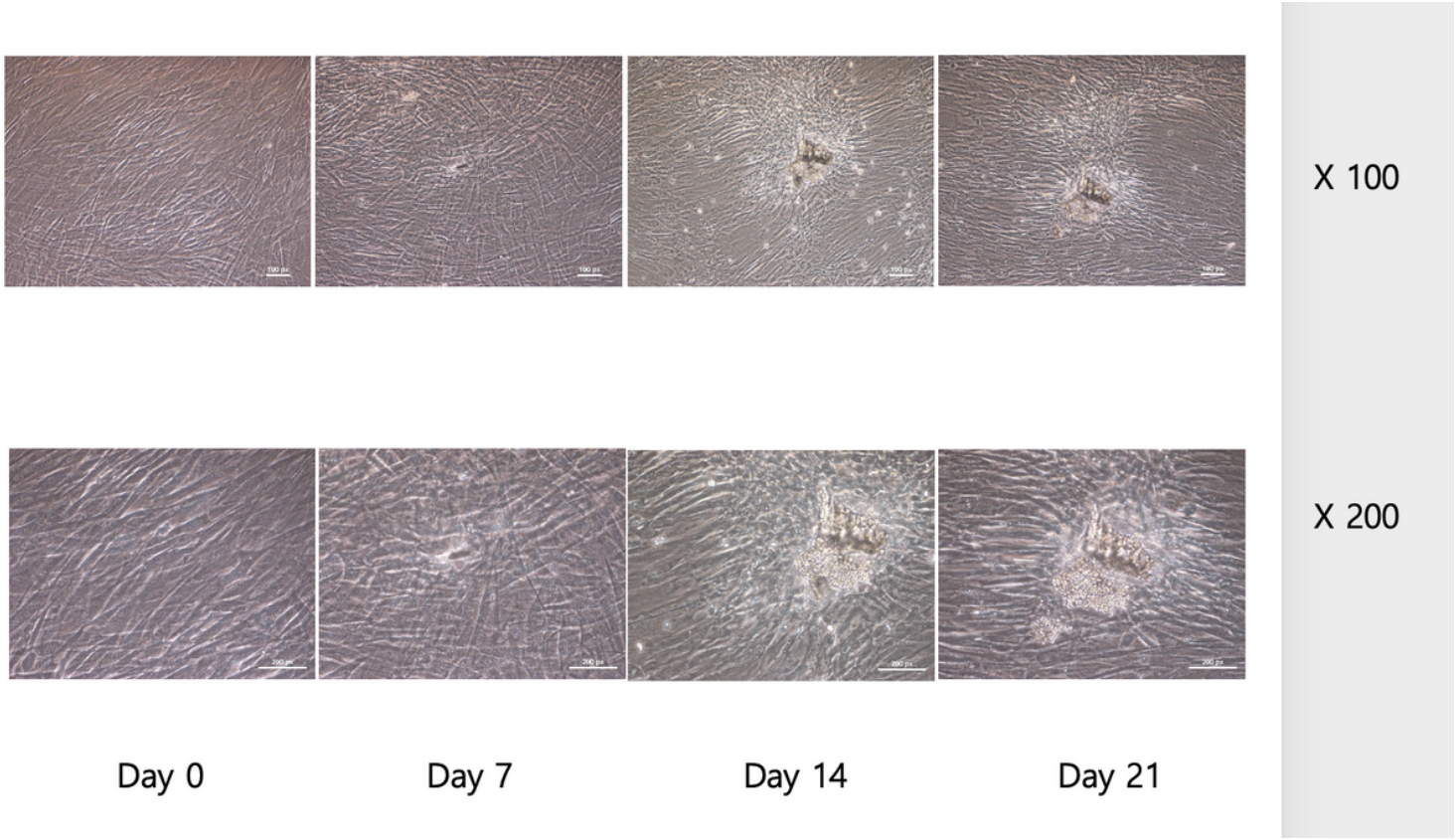


Figure 1

Light microscopy of differentiated adipose-derived stem cells (ADSCs). Morphologies of ADSC observed by light microscopy during differentiation (×100 and ×200 magnification). Arrow, nodule-like shape representing possible parathyroid gland structure.



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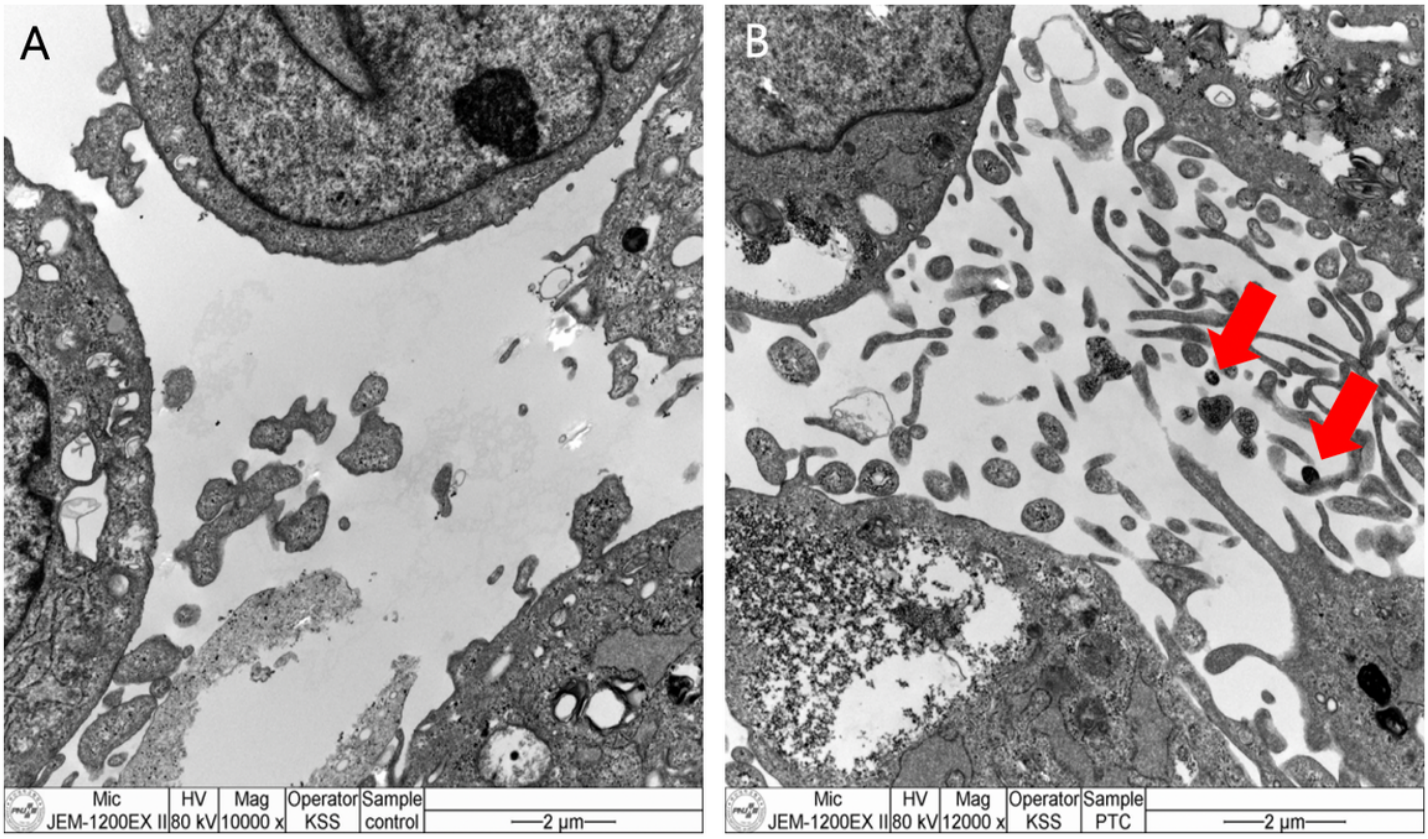
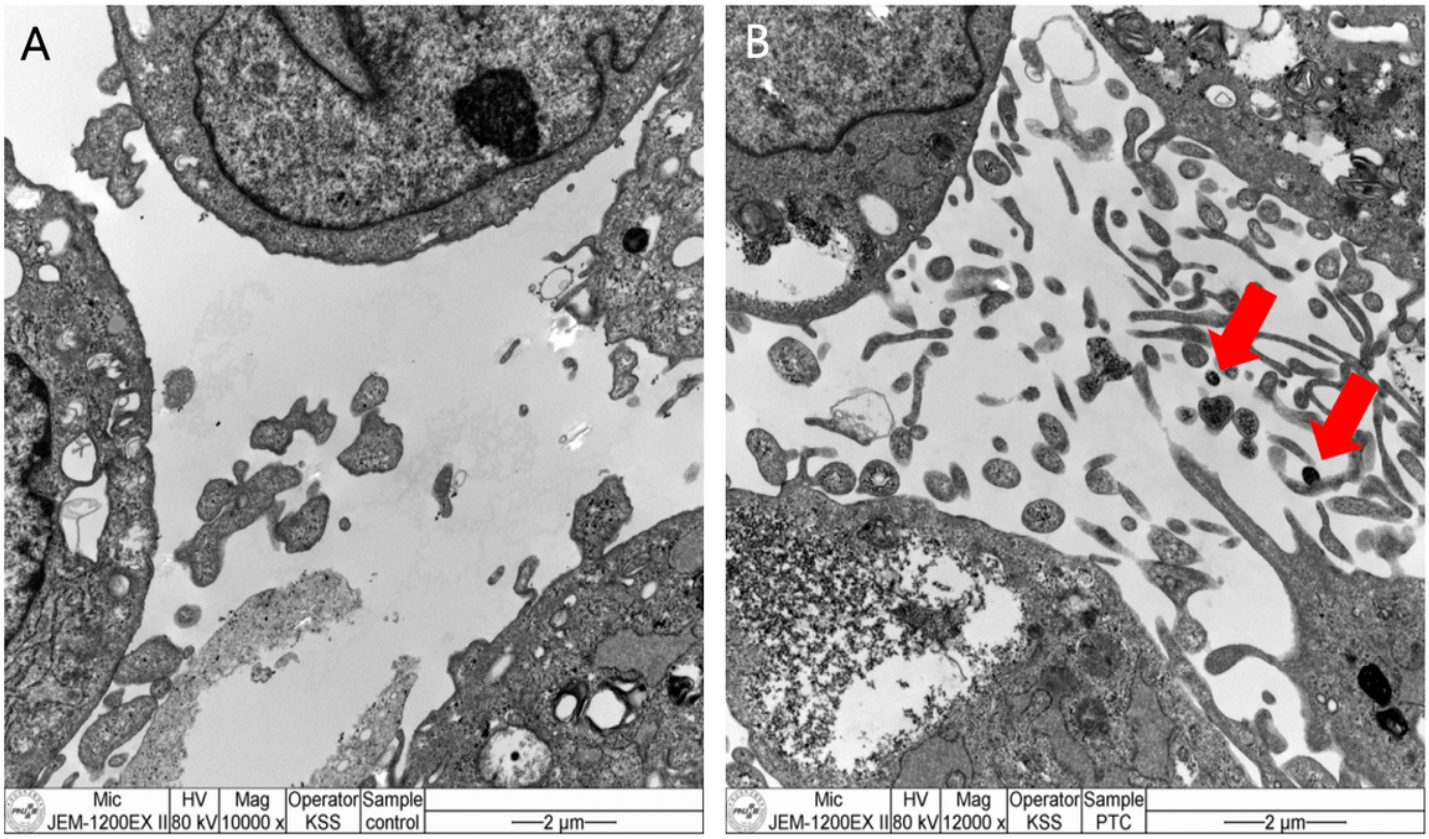


Figure 2

Transmission electron microscopy (TEM) of control (A) and differentiated adipose-derived stem cells (ADSCs) (B). Ultrastructural images of ADSC without or with differentiation captured by TEM. Distinctly dense spots (arrows) are found in the lumen of ADSC, while no such structures are found in the control.



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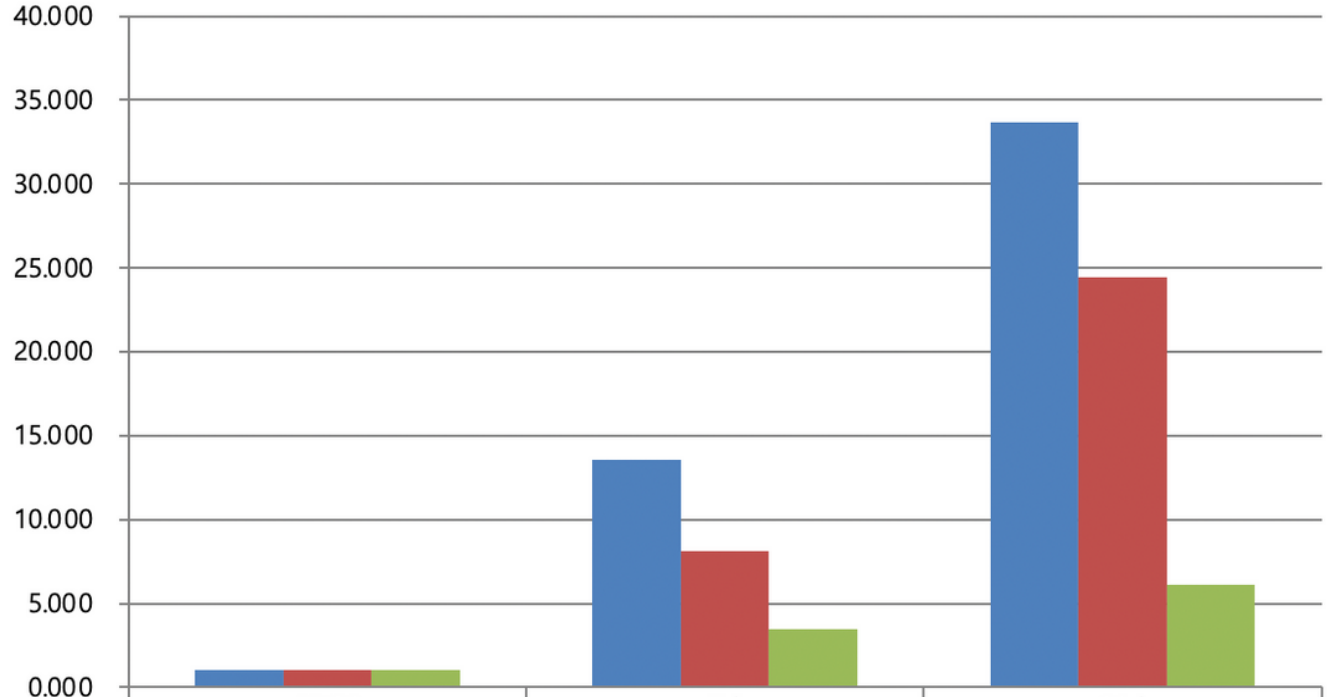
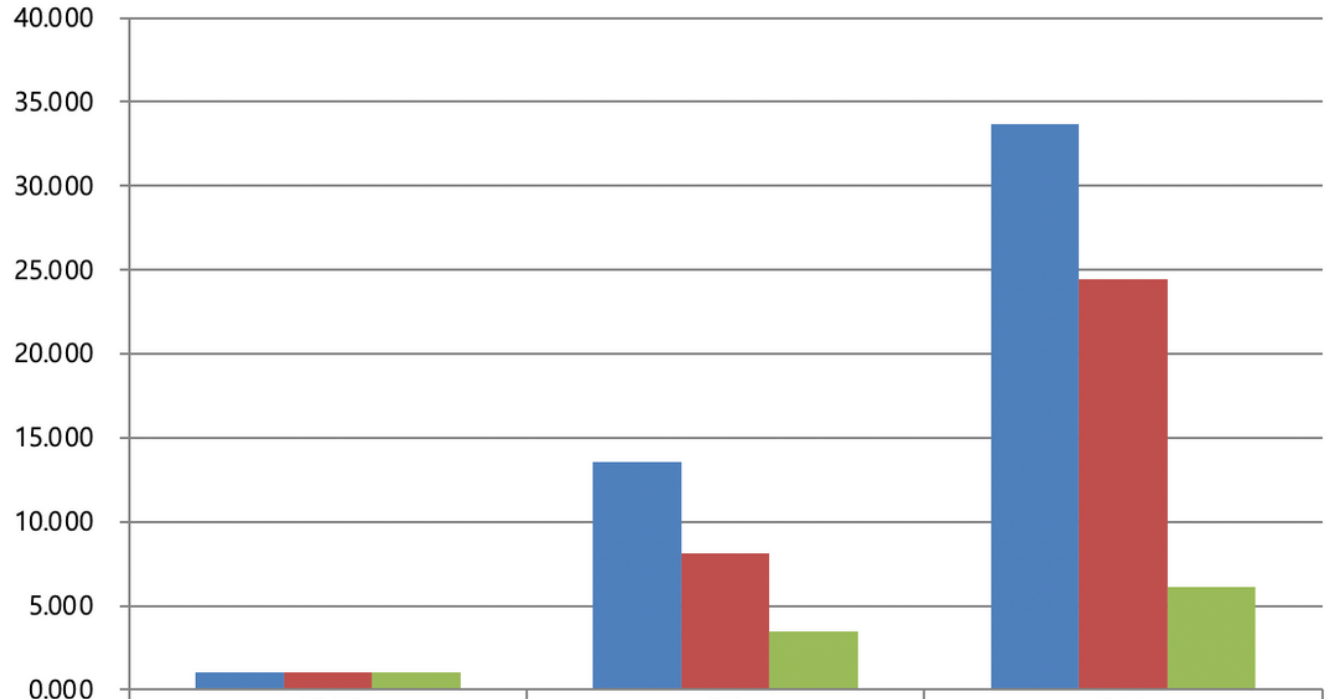


	ADSC control	7d diff	21d diff
PTH	1.000	13.593	33.691
GCM-2	1.000	8.103	24.428
CCL21	1.000	3.437	6.098

Figure 3

Real-time quantitative polymerase chain reaction (RT-qPCR) of parathyroid markers [control and differentiated adipose-derived stem cells (ADSC)]. RT-qPCR analysis reveals a significant increase in mRNA expression of the parathyroid markers PTH, GCM-2, and CCL21. Compared with the β-actin housekeeping gene, PTH, GCM-2, and CCL21 are upregulated 13.59-fold, 8.10-fold, and 3.43-fold, respectively, on the seventh day of differentiation and 33.69-fold, 24.42-fold, and 6.09-fold, respectively, on the 21st day of differentiation.



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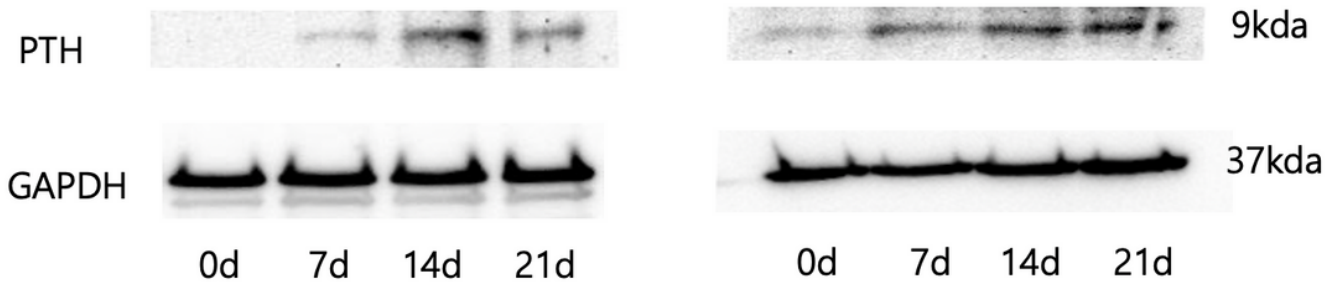

Figure 4

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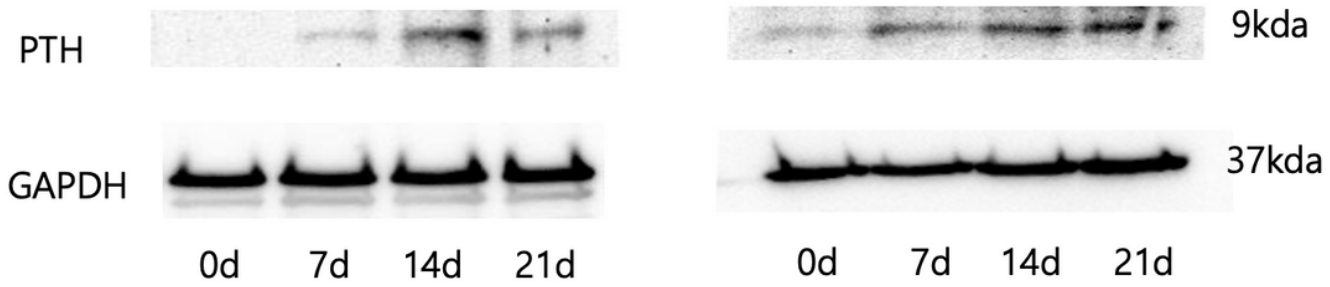

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