Intracellular Remodeling and Accumulation of Aberrant Lysosomes in Differentiation of Tonsil-Derived Mesenchymal Stem Cells into Parathyroid-Like Cells

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Received: 30 July 2016/Revised: 19 September 2016/Accepted: 26 September 2016/Published online: 3 July 2017
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Abstract Differentiation of mesenchymal stem cells (MSC) into a variety of cell lineages such as adipocytes, osteocytes, and chondrocytes is often accompanied up-regulation of autophagy. In our study, we demonstrated that the expression of autophagy-associated proteins (p-Beclin 1, LC3A, LC3B, p-AMPK, p-mTOR and ATG3, ATG7, and ATG12-5) over a period of time was hardly distinguishable from control tonsil-derived MSC (TMSC). Despite the unnoticeable difference in autophagy activation between differentiated TMSC (dTMSC) and the control (cTMSC), we reported significant changes in intracellular compositions in differentiated TMSC into functional parathyroid-like cells secreting parathyroid hormone (PTH). By using transmission electron microscopy (TEM), we observed accumulation of multivesicular bodies (MVB) comprising small, degraded compartments densely accumulated as dark granular or amorphous clumps, multilamellar bodies and lipid droplets in dTMSC. However, no such structures were found in cTMSC. These results suggest that differentiation of TMSC into parathyroid-like cells producing PTH hormone is hardly dependent on autophagy activation in the beginning of our conditions. Furthermore, our results of intracellular remodeling and accumulated endo-lysosomal storage bodies in the later stages of TMSC differentiation present a possible role of the structures in PTH secretion.

Keywords Tonsil-derived mesenchymal stem cells · Autophagy · Parathyroid-like differentiation · Multivesicular bodies · Multilamellar bodies

1 Introduction

Macroautophagy (autophagy hereafter) is a self-destructive mechanism through a regulated process of degradation and recycling of cytoplasmic components for cellular survival, apoptosis and energetics. Autophagy is mediated by autophag-

gic vacuoles (AV) that are developed in stages of maturation. Autophagosome formation are triggered by ATG proteins such as ATG3, ATG5, ATG7 and ATG12, LC3 membrane proteins, and Beclin 1, which are required for vesicle nucleation and expansion of autophagosome membrane [1–4]. After initial autophagosomes sequester unnecessary, dysfunctional or

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abnormal cytoplasmic contents, they can go through maturation either via fusion with endosomes or directly fuse with lysosomes for further hydrolytic degradation.

Autophagy can be triggered in response to various conditions including energy deprivation (starvation), damaged mitochondria, accumulation of unnecessary cytoplasmic organelles or protein, tumors, hypoxia, aging, bacteria, and viral infection [2, 5]. In stem cells, it is shown that autophagy switch is modulated by various pathways including mTOR, miR-17, miR-20, miR-93, miR-106, PTEN, P53, DRAM, HDAC6, TFEB, AMPK, and Akt [5–8]. Autophagy activation is observed during osteogenesis, adipogenesis and fibrogenesis of MSCs, and it is proposed that autophagy promotes maintenance and self-renewal and prevents apoptosis of MSC during differentiation [6, 8–10]. Furthermore, autophagy is highly coordinated or modulated by various signals, and therefore it is suggested that balance of autophagy in early stage of MSC differentiation dictates overall stemness, commitment and efficiency of MSC differentiation [5, 10, 12, 13].

Previously, we obtained multipotent mesenchymal stem cells (MSC) from palatine tonsillar tissues (TMSC) and successfully differentiated them into multiple mesoderms (adipocyte, osteocytes, chondrocytes, and tenocytes) [14–16]. Most recently, we differentiated TMSC into fully functional parathyroid-like cells, which were capable of producing PTH in a parathyroidectomized (PTX) rat model [17]. Hence, the role of autophagy in production and secretion of PTH during differentiation of TMSC into parathyroid-like cells has not been studied yet.

We here examined whether autophagy is manifested during the differentiation of TMSC into parathyroid-like cells. In our study, we found little evidence of up-regulated autophagy activation. Expression of autophagy-related proteins (p-Beclin 1, LC3, p-AMPK, p-mTOR, and ATG 3, ATG7, and ATG12-5) that are involved in autophagosome formation was similarly at basal level of the control in our conditions. Instead, we revealed intracellular remodeling accompanied by formation of various endo-lysosomal bodies such as multivesicular bodies (MVB) and multilamellar bodies (MLB) in dTMSC by ultrastructural analysis. Based on our results, parathyroid-like differentiation of TMSC is morphologically characterized with formation and accumulation of endo-lysosomal bodies, with little evidence of autophagy up-regulation under our conditions.

2 Materials and methods

2.1 Isolation and culture

TMSC isolation and culture were conducted as previously described [17]. Palatine tonsils were obtained from

tonsillectomy of three patients (2 boys and 1 girl, mean age 7.6 years) in 2013 and 2015 at the Department of Otorhinolaryngology – Head and Neck Surgery, Ewha Womans University Medical Center (EWUMC, Seoul, Korea). After tonsillectomy, tonsil tissues were minced and enzymatically digested in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Daegu, Korea) containing 210 U/mL collagenase type I (Invitrogen) and 10 mg/mL DNase (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C. After filtering the digested tissues by a wire mesh, the cells were washed twice with DMEM containing 20% fetal bovine serum (FBS) and once with DMEM containing 10% FBS.

Ficoll-Paque Plus (GE Healthcare, Little Chalfont, UK) density gradient centrifugation was performed to obtain mononuclear cells. Obtained cells (1×10^8 cells) were cultured in a T150 flask with DMEM containing 10% FBS and antibiotics (50 µg/ml streptomycin and 50 IU/ml penicillin; Gibco, Grand Island, NY, USA). While non-adherent cells after 48 h were washed out, adherent mononuclear cells (considered TMSC) were cultured in new culture medium. All TMSC used in this experiment were passage 5–8.

2.2 Differentiation of TMSC into parathyroid-like cells

TMSC were differentiated into parathyroid-like cells using the modified Bingham protocol [18] as previously described [14]. TMSC cultured with 90% confluency were treated with differentiation medium containing activin A (100 ng/mL) and soluble sonic hedgehog (Shh, 100 ng/mL) at dilution of 1:1000 for 7 days. Differentiation medium was changed every 3–4 days in this experiment.

2.3 Fluorescence-activated cell sorting

For cell-surface marker expression profiling, TMSC were cultured for 3 days at 80% confluency. TMSC (1×10^5 cells) were cultured and stained selectively with positive and negative stem cell markers based on the guidelines proposed by the International Society of Cellular Therapy [19]. The stained TMSC were analyzed by a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA). The antibodies used for flow cytometry were hematopoietic cell markers CD14-phycoerythrin (PE), CD34-PE, CD45-fluorescein isothiocyanate (FITC) for negative stem cell selection; endothelial marker CD31-FITC for negative selection; and primitive cell markers CD73-PE, CD90-FITC and CD105-FITC for positive selection (BD Biosciences, San Diego, CA). Non-specific binding selection was also measured by using isotype control antibodies IgG1 and IgG2a (Becton Dickinson).

2.4 Measurement of secreted intact PTH (iPTH) concentration

To measure secreted iPTH protein concentration, conditioned media (CM) of cTMSC and dTMSC were collected. CMs were separately collected every day for seven days of differentiation and filtered by a 0.2 µm syringe filter. Secreted iPTH concentration was measured by electrochemical luminescence immunoassay (ECLIA) using an Elecsys PTH kit (Roche Diagnostics, Mannheim, Germany).

2.5 Confocal laser microscopy for PTH Expression and lysosomes detection

Confocal microscopy was used to determine intracellular localization of synthesized PTH and lysosomes during TMSC differentiation. TMSC were seeded on a single cover glass placed in each well of a 6-well plate (1×10^6 cells/cover glass). TMSC were treated with differentiation medium and incubated for seven days. Then, cTMSC and dTMSC at day 7 were used for confocal microscopy. The level of PTH was measured using an anti-PTH (1:100, Abnova, Seoul, Korea), followed by their corresponding fluorescent secondary antibodies.

Lysosomes were fluorescently detected by fluorescent dye LysoTracker® Red DND-99 (Molecular Probes, Eugene, OR, USA). LysoTracker dye (75 nM) was added to each well plate containing a cell-covered cover glass and incubated at 37°C for 90 min. After washing with DPBS three times, each cover glass was fixed with 4% paraformaldehyde (PFA) for 10~30 min at room temperature. They were washed with DPBS again. TMSC were subjected to confocal analysis using a Zeiss LSM 800 Confocal Laser Microscope (Carl Zeiss, Oberkochen, Germany). Relative fold change of LysoTracker was quantitatively measured using Image J program (National Institutes of Health, Bethesda, MD).

2.6 Transmission electron microscopy

To analyze the intracellular organelle changes of TMSC during the differentiation, cTMSC and dTMSC were harvested and fixed with 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) for 24 h. They were then post-fixed with 1% OsO₄ in 0.1 M PBS (pH 7.4) for 1 h at room temperature. TMSC samples were completely dehydrated before they were embedded in epoxy resin. Ultrathin TMSC sections were counterstained with lead citrate and uranyl acetate, and then ultrastructural analysis was performed by using a transmission electron microscope (JEM-1400, JEOL USA Inc., MA, USA).

2.7 Western blot analysis

Both cTMSC and dTMSC at day 0, day 1, day 3 and day 7 were collected and total protein level at each day was measured. TMSC were lysed and extracted using 1mM of RIPA buffer containing Protease Inhibitor Cocktail™ (Roche Molecular Diagnostics, IN, USA), 1mM of PMSF, 1mM of NaF, 1mM of Na₃VO₄, and 1mM of Glycerol-2-phosphate.

BCA protein assay kit (Sigma-Aldrich) was used to measure the protein concentrations. Then, TMSC proteins (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane. The blots were subsequently probed using the appropriate antibodies directed against p-Beclin 1 (1:1000, Cell Signaling Technology, Danvers, MA, USA), LC3A (1:1000, Cell Signaling Technology), LC3B (1:1000, Cell Signaling Technology), p-AMPK (1:1000, Cell Signaling Technology), AMPK (1:1000, Cell Signaling Technology), p-mTOR (1:1000, Cell Signaling Technology), chromogranin A (1:1000, CHGA, Ab Frontier, Seoul, Korea), ATG 3 (1:1000, Cell Signaling Technology), ATG7 (1:1000, Cell Signaling Technology), ATG12 (1:1000, Cell Signaling Technology), and Actin (1:3000, Ab Frontier). The level of Actin expression was used as an internal control. Band images obtained upon western blot analysis were quantified using the Image J software.

2.8 Statistical analysis

Statistical analyses were performed using SPSS (Version 21.0). Data were expressed as means ± standard deviation (S.D.) and statistically significant differences between experimental groups were analyzed using a Student's *t*-test with *P*-values <0.05 considered significant (*).

3 Results

3.1 MSC surface phenotype of TMSC

To examine whether the TMSC possess the mesenchymal properties, we performed FACS using various MSC surface markers. FACS results showed that TMSC expressed a very minimal expression of the hematopoietic cell markers CD14, CD34, and CD45 or endothelial cell maker CD31 (<0.3%) and positive expression of the primitive cell makers CD73, CD90, and CD105 at a higher rate (>97%) (Fig. 1). The FACS analysis revealed that TMSC appropriately expressed the essential MSC-specific surface phenotypes.

Fig. 1 Phenotypic expression of TMSC. The cells were labeled with antibodies against CD14, CD34, CD45, CD31, CD73, CD90 and CD105 by using FACS. *TMSC* tonsil-derived mesenchymal stem cells

3.2 Differentiation of TMSC into parathyroid-like cells secreting iPTH

To confirm the TMSC differentiation into parathyroid-like cells, we measured released iPTH concentrations from cTMSC and dTMSC over 7 days. CM from cTMSC and dTMSC were collected each day throughout seven days of differentiation, and secreted iPTH level was measured by using an ECLIA. As a result, we observed an increasing release of iPTH from dTMSC from day 0 and a peak level of ~30 pg/ml till day 7. On the other hand, iPTH level from cTMSC was substantially lower, and it leveled off after day 3 (Fig. 2A).

Moreover, immunocytochemistry analysis successfully confirmed that PTH was more localized in dTMSC than the

cTMSC at day 7 (Fig. 2B). Our results show that TMSC could successfully differentiate into parathyroid-like cells and secreted a high level of PTH than the control.

3.3 Differentiation of TMSC showed little evidence of autophagy up-regulation

To investigate whether there was autophagy activation in early TMSC differentiation, we collected the cTMSC and dTMSC at different time points (day 0, 1, 3, and 7) and then measured a coordinated time-dependent expression of autophagy markers such as p-Beclin 1 (Ser93/96), LC3A, LC3B, p-AMPK (Thr172), and p-mTOR (S2448) (Fig. 3A).

Fig. 2 Production and secretion of PTH from differentiated TMSC. **A** Daily secreted concentration of iPTH from undifferentiated (cTMSC) and differentiated TMSC (dTMSC) was quantitatively measured by ECLIA. **B** Immunofluorescence confocal microscopic analysis of PTH was performed in cTMSC and dTMSC at day 7. Results are expressed as the means $\pm$ SD (* $p < 0.05$)

We found a minimal difference in time-dependent autophagic expression between cTMSC and dTMSC. We observed gradually increasing expression of p-Beclin1, which involved in autophagosome nucleation and membrane expansion, in time-dependent manner in both cTMSC and dTMSC. We also observed an increasing expression of both LC3A-II and LC3B-II in a time-dependent manner. Conversion of LC3A/B-I to LC3A/B-II has been used as a conventional marker of autophagosome formation since during initial autophagy, cytosolic LC3-I is lipidated by conjugation with phosphatidylethanolamine (PE) and converted into LC3-II, which is membrane-bound in autophagosomes. In our study, the time-dependent increase of LC3A-II was more significantly measurable than that of LC3B-II (Fig. 3A). We measured relative fold change of LC3A-II/Actin throughout the time period in respect to each initial value at day 0 (Fig. 3B). Both cTMSC and dTMSC showed greater expression of LC3A-II in time-dependent manner. In dTMSC, LC3A-II/Actin at day 7 was about 4-fold greater than that of day 0 (Fig. 3B).

The p-AMPK expression increased in both cTMSC and dTMSC in a time-dependent manner meanwhile expression pattern of p-mTOR remained fairly stable.

To confirm whether autophagy activation was indeed driven in dTMSC, we additionally measured and compared protein expression of ATG3, ATG7 and ATG12-5, which are involved in LC3 conjugation process, between cTMSC and dTMSC. We demonstrated that ATG3, ATG7 or ATG12-5 expression did not fluctuated significantly over the time period when comparing the two groups (Fig. 3C). Despite, we found significant expression of PTH-related secretory granule marker CHGA at day 7 of dTMSC, validating that parathyroid-like differentiation was effective in seven days (Fig. 3A).

Overall, current results demonstrated that there was that autophagy up-regulation was hardly prevalent in early stages of TMSC differentiation compared to the control, and instead autophagosome formation was more profoundly activated in the later time period.

3.4 Accumulation of intracellular lipids and endo-lysosomal bodies in differentiated TMSC

In our conditions, there was little evidence for autophagy activation during differentiation. To investigate autophagy or autolysosomal flux in parathyroid differentiation of TMSC, we performed confocal analysis using LysoTracker, a fluorescent dye for labeling and tracking acidic vacuoles and organelles including lysosomes. Since we found that dTMSC released the maximum level of iPTH secretion and PTH expression at day 7, we labeled and compared electron microscopic images of cTMSC and dTMSC at day 7. Furthermore, we performed transmission electron microscopic (TEM) analysis to determine whether AV or lysosomal vacuoles were formed during differentiation.

We demonstrated excessive lysosomal vacuoles in dTMSC (Fig. 4). LysoTracker efficiently labels lysosomes (pH ~ 4.5) and also detects some mildly acidic interiors of organelles such as late endosomes (pH ~ 5.5) or autolysosomes [20]. Furthermore, our results showed that lysosomal vacuoles in the dTMSC were expressed almost ubiquitously within a cell with slightly less fluorescent intensity, compared to that of cTMSC that showed few highly fluorescent, acidic vacuoles that localized explicitly around the nucleus in the control (Fig. 4A). The overall fluorescence intensity of LysoTracker-positive vesicle was about 1.5 times greater in dTMSC than cTMSC (Fig. 4B).

We then preformed ultrastructural analysis of cTMSC and dTMSC by using a TEM. Our analysis exhibited some dramatic differences in intracellular conditions of dTMSC from cTMSC (Fig. 5). The most evident difference was formation of various lysosomal structures (white arrows in Fig. 5B). Many small, degraded compartments densely

Fig. 3 Western blot analysis of autophagy-related proteins in undifferentiated and differentiated TMSC. **A** Expression of p-Beclin1, LC3AI, LC3A-II, LC3B-I, LC3B-II, p-AMPK, AMPK, p-mTOR, mTOR, and CHGA were measured, and expression of Actin was used as an internal control. **B** Relative fold change of LC3A-II/Actin was

quantitatively measured using an Image J program. **C** Expression of ATG3, ATG7, ATG12-5 were measured. Results are expressed as the means $\pm$ SD (* $p < 0.05$ when compared day 0 vs. each day in cTMSC and dTMSC)

accumulated as dark granular or amorphous clumps, known as multivesicular bodies (MVB), were accumulated only in dTMSC (white arrows in Fig. 5B) compared to cTMSC (Fig. 5A). MVB are composed of electron-dense multiple vesicles, which are sequestered individually in an intact cytoplasm. Overall, MVB demonstrated the features that give similarity or resemblance to that of late endosomes. MVB can be either fused directly with plasma membrane, releasing luminal vesicles called exosomes to extracellular space via exocytosis, or digested by lysosomes. Furthermore, the number of lipid droplets in cytoplasm was slightly greater in dTMSC than cTMSC (white arrow heads in Fig. 5B). These results together suggest that there was greater accumulation of stored lipids, as in form of MLB or lipid droplets, in dTMSC. The overall presence of MVB was quantified, and the number of MVB was significantly greater in dTMSC than cTMSC (Fig. 5C).

Moreover, a number of lipid-rich multilamellar bodies (MLB) were accumulated more in dTMSC (white arrows in Fig. 6C) than cTMSC (Fig. 6A, B). In general, MLB are defined as membrane bound cytoplasmic organelles

presenting at least three distinct circumferential concentric membrane lamellae and known to be rich in lipids [21]. Interestingly, in dTMSC, we found some cases as if MLB were fused with MVB (black arrows in Fig. 6D). The overall presence of MLB was also quantified, and it was evidently detected only in dTMSC (Fig. 6E).

In general, the abnormal lysosomal bodies including MVB and MLB within a cell were accumulated evidently more in dTMSC than cTMSC while those structures were hardly identified in cTMSC (Figs. 5C, 6E).

4 Discussion

The role of autophagy during differentiation of MSCs has been extensively investigated, however a little is known about autophagy and its related protein expressions during the differentiation of TMSC into parathyroid-like cells. Here, we revealed some notable changes in intracellular compositions such as accumulations of the various endo-lysosomal bodies in dTMSC with successful secretion of PTH.

Fig. 4 Confocal microscopic analysis of lysosomes or mildly acidic vacuoles in TMSC at day 7 by using LysoTracker.

A Lysosomal vacuoles in the dTMSC were expressed almost ubiquitously within a cell with slightly less fluorescent intensity, compared to that of cTMSC. **B** Relative fold change of LysoTracker was quantitatively measured using Image J program.

Despite that it has been previously reported in other MSC differentiation studies, our western blot results give little evidence for autophagy up-regulation in early days, as the expression of autophagy-related proteins was not significantly different from the control. Still, time-dependent expression of LC3A-II protein at day 7 might indicate either increasing formation of autophagosome or accumulation of LC3A-II proteins due to impairment in autophagy flux. Autophagy flux is often mediated by fusion of autophagosome with endosomes or lysosomes into amphisomes or autolysosomes, and inhibition of autophagy flux often results in excess of matured autophagosomes and thereby accumulation of LC3A-II protein [7, 22]. Instead, our study exhibited that autophagy activation in favor of autophagosome formation is more likely prevalent in later stages of differentiation with its significant intracellular organelle remodeling.

Intracellular modifications during the parathyroid-like differentiation of TMSC were explicitly demonstrated with

accumulation of endo-lysosomal organelles such as MVB and MLB with increased number of lipid droplets. Although the role of MVB and MLB in dTMSC needs to be studied furthermore, accumulation of the various endo-lysosomal bodies is likely associated with parathyroid-like differentiation of TMSC and secretion of PTH.

Previously, it was reported that functions of lysosomes sometimes vary and they are specialized differentially based on their locations in a cell [23, 24]. For instance, perinuclear lysosomes are known to be involved in proteasomal degradation via endocytic/phagocytic and autophagic pathways while membrane proximal lysosomes have higher pH level and are likely engaged in lysosomal vesicular trafficking and exocytosis [24]. The difference in distribution and pH level of lysosomal bodies in dTMSC compared to cTMSC may illustrate some possible changes in endo-lysosomal pathways such as loss of lysosomal enzymes or function, defective lysosomal trafficking or maturation via fusion with late endosomes, MVB or

Fig. 5 Accumulation of multivesicular bodies and lipid droplets in TMSC. **A** Ultrastructure of cTMSC and **B** dTMSC at day 7 were analyzed by using TEM. Accumulation of MVB (white arrows) and lipid droplets (white arrow heads) was found in **B** dTMSC.

C Accumulation of MVB was quantitatively measured by using Image J program. Scale bar 5 μ m. N.D. not detected. TEM transmission electron microscopy, MVB multivesicular bodies

autophagosomes, and accumulation of undegraded macromolecules in lysosomes [25–27].

The modification in endo-lysosomal pathways could arise from defects in maturation of lysosomal bodies, interactive trafficking between lysosomal bodies and other organelles and pH homeostasis of lysosomes. Overall, the confocal analysis refers to accumulation of lysosomal bodies that might be due to impairment of lysosomal trafficking and activity in dTMSC at day 7 (Fig. 4). Although the role of aberrant endo-lysosomal structures during parathyroid differentiation of TMSC is not explicitly clear, increasing evidence highlights that they make some physiologically functional features and contributions in secretory pathways [21, 28–30]. Furthermore, lipid-rich MLB, as well as lipid droplets (Fig. 5B), are known to be storage of Ca^{2+} , which is a key regulator of lysosomal pH, lipid homeostasis, vesicle trafficking, membrane dynamics and exocytosis [27, 31]. We propose that the formation of

endo-lysosomal bodies is very likely coupled with secretion of PTH via both intracellular and extracellular Ca^{2+} -dependent manners in our conditions. We previously reported that the PTH expression was regulated by extracellular Ca^{2+} levels [17]. To this end, the formation of endo-lysosomal body in dTMSC can be explained as a feature of intracellular Ca^{2+} regulation or a mechanism of PTH secretion in dTMSC.

Our finding might draw a morphological similarity to parathyroid gland. In fact, abundant lipid droplets are present in parathyroid chief cells, where PTH is produced and secreted, and it was evidenced by a previous study that the quantity of lipid droplets in normal parathyroid gland is reported to be greater than in abnormal cases including adenoma (benign tumor), hyperplastic (enlargement) or carcinomatous glands [32]. Based on this information, greater number of lipid droplets in dTMSC (Fig. 5B) might possibly refer to alterations in endo-lysosomal pathways,

Fig. 6 Accumulation of aberrant lysosomal bodies in TMSC. **A, B** Ultrastructural analysis of cTMSC and **C, D** dTMSC at day 7 by using TEM. Accumulation of aberrant lysosomal bodies such as MLB (white arrows) was found in **C** dTMSC. In addition, fusion of MLB

and MVB (black arrows) was observed in **D** dTMSC. **E** Accumulation of MLB was quantitatively measured by using Image J program. Scale bar 2 μ m. *N.D.* not detected. *TEM* transmission electron microscopy, *MLB* multilamellar bodies, *MVB* multivesicular bodies

specifically in lipid metabolism or lipid trafficking. Thereby, morphology of the aberrant lysosomal storage bodies, specifically MLB, may clarify the state of intracellular Ca^{2+} regulation and homeostasis during parathyroid-like differentiation. Overall, it may be highly relevant that MVB or MLB formation during TMSC differentiation indicate some significant changes in endo-lysosomal pathways encompassing lysosomal Ca^{2+} homeostasis or lipid storage. Excessive storage of lipids or Ca^{2+} in lysosomes is highly characterized with the morphology of multilamellar, eccentric inclusions. That being said, main function of MLB in dTMSC is possibly to capture excessive Ca^{2+} inside the lysosomal lumen, lowering the cytosolic Ca^{2+} concentration, and therefore up-regulating secretion of PTH.

Taken together, we revealed that parathyroid-like differentiation of TMSC is merely mediated by autophagy in initial stages in our conditions, but regardless we showed dramatic intracellular modifications including accumulation of the aberrant lysosomal storage bodies or lipids, which are highly regulated in endo-lysosomal pathways. Our study provides morphological evidence for manipulations in endo-lysosomal pathways during parathyroid-like differentiation of TMSC, presumably in a manner that Ca^{2+} -dependent exocytosis is sophisticatedly manipulated in order to enhance secretion of PTH in dTMSC. Still, further studies are necessary to elucidate how TMSC differentiation is appropriately regulated by autophagic and endo-lysosomal pathways. Our study may suggest a broader scope of a possible cross-talk between autophagy and endo-

lysosomal pathways during TMSC differentiation into parathyroid-like cells.

Acknowledgements This study was supported in part by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning NRF-2013R1A1A3007591 and 2017R1A2B4002611 by the Grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI14C-1557). This work was supported by the research grant of the Chungbuk National University in 2016.

Compliance with ethical standards

Conflicts of interest All authors have no conflict of interest.

Informed consent Informed written consent was obtained from legal guardians of all patients participating in this study.

Ethical statement The study protocol was approved by the EWUMC Institutional Review Board (IRB #ECT11-53-02).

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