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MicroRNA-211 and autophagy-related gene 14 signaling regulate osteoblast-like cell differentiation of human induced pluripotent stem cells

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ABSTRACT

MicroRNAs (miRNAs) have been the subject of recent attention as key regulatory factors in cell differentiation. In the current study, to explore the early signaling cascade of osteogenic differentiation of human induced pluripotent stem (hiPS) cells, we investigated miR-211 regulation and autophagy-related gene (Atg) signaling in osteogenic differentiation. In addition to reciprocal strong induction of miR-211 expression in differentiated cells following osteogenic differentiation, we found abundant Argonaute 3 bound to miR-211. There were also dramatic increases in the mRNA and protein levels of Atg14 together with increases in the amount of autophagosomes as well as autophagic fluxes. While transfection of a miR-211 inhibitor abrogated the induction of Atg14, autophagy events, osteoblast differentiation markers, and induction of calcification were suppressed markedly. Treatment with small interfering RNAs against Atg14 also suppressed the osteogenic differentiation medium (ODM)-induced increase in osteogenic differentiation. The osteogenic phenotype was inhibited by chloroquine (an autophagy inhibitor), but increased after treatment with rapamycin (an autophagy inducer). Taken together with our previous findings, we have revealed a unique sequential cascade involving miR-211 and Atg14 in ODM-induced differentiation of hiPS cells into osteoblast-like cells at a relatively early stage.

1. Introduction

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression at the post-transcriptional level by degrading mRNA or inhibiting translation [1]. Accumulating data suggest that miRNAs play important roles in regulating stem cell self-renewal, differentiation, and pluripotency [2,3]. In addition, miRNAs regulate proliferation, senescence, migration, and survival of mesenchymal stem cells (MSCs) [4]. Previous studies have shown that miRNA-211 (miR-211) plays key roles in cancer cell migration. However, it can either promote [5–7] or suppress tumor cell migration [8], depending on the cell types and pathological conditions.

Differentiation of MSCs into mature osteoblastic cells and regulation of osteoblast functions involve highly regulated processes mediated by a large number of hormones and locally produced growth factors. Regulatory factors of the osteoblastic phenotype include essential transcription factors Runx2/Cbfa-1 and Osterix/SP7 [9,10], and major signaling pathways including bone morphogenetic protein (BMP), Wnt, and notch, [11–13] as well as other growth factor-mediated kinase signaling pathways [14]. Emerging evidence has revealed an additional level of regulation mediated by small non-coding, single-stranded miRNAs [15]. Li et al. found high expression of miR-188 in aged mice and humans, and that it regulates bifurcation of bone marrow stromal cells (BMSCs) differentiating into osteoblasts and adipocytes [16]. miR-204 and its analogue, miR-211, have been reported to act as vital negative regulators of Runx2 to promote adipogenesis and suppress osteogenesis in bone morrow-derived MSCs [17]. Because the roles of miRNAs in regulating differentiation of stem cells, including induced pluripotent stem (iPS) cells, have not been studied at present, which miRNAs regulate osteogenic differentiation remains unknown. Therefore, we hypothesized that specific miRNAs play an essential role during osteogenic differentiation, especially in human iPS (hiPS) cells, as positive regulators.

Autophagy is a genetically regulated and dynamic process associated with the formation of autophagosomes, double membrane cytoplasmic vesicles that engulf cellular components [18]. Depending on the cellular context and stimulus, the outcome of autophagy promotes either cell survival/proliferation or death [19–21]. An increasing number of studies have shed light on the importance of autophagy, particularly autophagy-related gene (Atg), in a wide range

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of physiological processes and human diseases [19–21]. However, the physiological function of autophagy, especially in osteoblastic differentiation of hiPS cells, is not well defined.

We have previously performed experiments using purified osteoblast-like cells derived from human skeletal muscle stem cells (hSMSCs) [22–24]. These osteoblast-like cells are an excellent in vitro model to examine the mechanisms of wound healing involving cell differentiation and/or proliferation in diseased areas such as inflammatory sites in rheumatoid arthritis. Although hiPS cells have potential use in regenerative medicine, there are no reports on osteoblastic differentiation of hiPS cells.

We explored the early signaling cascade of osteogenic differentiation and examined whether miRNA and Atg signaling are associated with osteogenic differentiation. For the first time, we show that miR-211 upregulates Atg14 expression in osteoblast-like cells, leading to enhanced differentiation of hiPS cells.

2. Materials and methods

2.1. Cell culture

The hiPS cell line 201B7 [25,26] was purchased from the Riken Cell Bank (Ibaraki, Japan) and maintained as described previously [26,27]. Briefly, the cells were cultured in flasks or plates coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ) in ReproFF medium (ReproCELL, Yokohama, Japan) at 37 °C with 5% CO_2 in a humidified incubator. At confluency, the cells were rinsed with phosphate-buffered saline (PBS) and harvested with Accutase (Innovative Cell Technologies, San Diego, CA). We used 25-cm^2 flasks (Asahi Techno Glass, Funabashi, Japan) to culture the cells, and observed the cells under a BZ-9000 microscope (Keyence, Osaka, Japan).

2.2. Osteogenic differentiation

Osteogenic induction was performed as described previously [28,29]. Briefly, the formation of embryoid body (EB)-like structures by hiPS cells was carried out using the hanging drop method based on a previously described protocol [30]. Cell aggregates were pooled in non-adherent culture dishes (Sumilon dish; Sumitomo Bakelite co., Ltd, Tokyo, Japan) to generate EBs that were cultured in suspension with 1×10^{-7} mol/L retinoic acid (RA; Sigma-Aldrich, St. Louis, MO) for 3 days. Then, the RA-treated cells ($1.5\times10^5/cm^2$) were cultured in osteogenic differentiation medium (ODM) supplemented with 1×10^{-7} M dexamethasone (Sigma-Aldrich) and 1.8 mM inorganic phosphate (Taihei Chemical Industrial Co., Ltd., Osaka, Japan) for 7 days. Up to day 21 of culture, these differentiated cells displayed osteoblast-like physiological characteristics including a calcification activity that was detected by alkaline phosphatase (ALPase) activation and Alizarin Red S (ARS; Sigma-Aldrich) staining.

2.3. ALPase activity assay

Cellular ALPase activity was measured using *p*-nitrophenol phosphate (Sigma-Aldrich) as a substrate [27,31]. Briefly, after washing, the cells were incubated in 150 μL ALPase buffer (Sigma-Aldrich) and 150 μL *p*-nitrophenol phosphate solution at 37 °C for 30 min with gentle shaking in the dark. Subsequently, 700 μL of a 3 M NaOH solution was added to each well to stop the reaction, and then 100 μL final solution was added to each well, followed by analysis at 405 nm in an ELISA reader (SH-1200 Lab, Corona Electric Co., Ltd, Ibaraki, Japan). The enzymatic activity was calibrated with a *p*-nitrophenol phosphate standard curve and expressed as micromoles of reaction product/minute/total protein obtained from protein quantification in each well.

2.4. ARS staining and quantification

Mineralization from hiPS cell-derived osteogenic cells was quantified using an ARS assay [27,31]. Briefly, after washing, the cells were immersed in a 40 mM ARS solution (pH 4.2) for 20 min at room temperature with gentle agitation. The solution was then removed, and the mineralized matrices were washed with flowing water. The morphology of mineralized matrices was observed and photographed under the BZ-9000 microscope. ARS staining was quantified using the method developed by Gregory and colleagues [32]. Briefly, after staining and washing, the samples were dried overnight, and then 0.8 μL of 10% (v/v) acetic acid (VWR International, Radnor, PA) was added to each well, followed by incubation at room temperature for 30 min with shaking. The loosely attached cells were scraped off with a cell scraper, transferred to a microcentrifuge tube, and vortexed for 30 s. Samples were heated at 85 °C for 10 min, followed by cooling on ice for 5 min. After centrifugation at 20,000*g* for 15 min, 200 μL of 10% (v/v) ammonium hydroxide (Sigma-Aldrich) was added. Finally, 100 μL of the supernatant was placed into a well of a 96-well plate and analyzed at 405 nm with the ELISA reader.

2.5. Real-time quantitative polymerase chain reaction (qPCR) analysis

Real-time qPCR was performed in triplicate in 96-well optical microtiter plates with ~25 ng RNA, 0.25 μL Quantitect RT Mix (Qiagen, Valencia, CA), 1.25 μL of 20× Primer/Probe Mix, and 12.5 μL Mastermix (Quantitect RT-PCR Kit; Qiagen) in a 25 μL reaction volume. The following primer/probe sets (Assays-On-Demand™; Applied Biosystems, Foster City, CA) were used: mouse Pou5f1 (OCT3/4), Mm03053917_g1 (human available); human osteocalcin (BGLAP), Hs01587814_g1; human osteopontin (SPP1), Hs00959010_m1; human RUNX2, Hs01047973_m1; human MIR141 (miR-141), Hs04406459_s1; human MIR200A (miR-200a), Hs04231538_s1; human MIR204 (miR-204), Hs04231468_s1; human MIR211 (miR-211), Hs04231471_s1; human miR-302a (hsa-miR-302a), MI0000738; human ATG5 (Atg5), Hs00355492_m1; human ATG7 (Atg7), Hs00197348_m1; human ATG10 (Atg10), Hs00919718_m1; human MAP1LC3A (LC3), Hs01076567_g1; human TFE3, Hs00232406_m1; human BECN1 (Beclin1).

Standards and samples were mixed with the PCR reagents and loaded into the 96-well microtiter plates that were then sealed with optical film (Applied Biosystems). The PCR cycling conditions were 30 min at 50 °C, 15 min at 95 °C, and then 40 cycles of 15 s at 94 °C and 60 s at 60 °C. A standard curve was used for relative quantitation of gene expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18 S rRNA were employed as housekeeping genes for normalization of the amount of total RNA in each sample. The amounts of target and endogenous reference mRNAs were determined from the appropriate standard curve. The amount of target mRNA was divided by the amount of endogenous reference mRNA to obtain a normalized target value. *Ct* (threshold cycle) values for the target and housekeeping genes were extrapolated from the standard curve to produce an arbitrary value of expression, the ratio of which (target gene/housekeeping gene) within each sample was plotted as the relative mRNA expression level.

2.6. Western blot analysis

OCT3/4, BGLAP, SPP1, Runx2, Argonaute (Ago) 1, Ago2, Ago3, Ago4, and Atg14 protein levels in cell lysates were determined by western blot analyses. Cells were cultured for 7 days with or without ODM and then lysed. The resulting protein samples were separated in 12% sodium dodecyl sulfate-polyacrylamide gels. Western blot analyses were performed using anti-Oct3/4, anti-osteocalcin, anti-OPN (osteopontin), anti-RUNX2, anti-Atg14, and anti-β-tubulin polyclonal

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