柑橘ポリフェノールの iPS 細胞オートファジーへの影響

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Effect of citrus polyphenols on autophagy in iPS cells

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Somatic cells age and die over time, stem cells, being immortal cellular lineages, can persist indefinitely. The autophagy function of the cell declines over time, and with this decline, damage accumulates. The question of how the stem cell can avoid transmitting the damage to the next generation is a significant problem. Here we identified hesperidin, a citrus flavonoid, that promotes autophagy in induced pluripotent stem cells (iPSCs). We found that iPS cells whose proliferation was arrested in the presence of lemon extract exhibited stimulation of autophagy, including protein hydrolysis. Strikingly, lemon extract inhibited glycosylation of iPS cells following arrest of proliferation. The key to this reduction is the activation of β-glucosidase, a glycosidase present in lysosomes. Activated lysosomes promote a metabolic shift that mobilizes glycoproteins for degradation and resets proteostasis by clearing glycoproteins. Lemon hesperidin may stimulate autophagy activity in iPS cells through the TGF signaling pathway, a cytokine that blocks growth signals.

KEYWORDS: polyphenol, iPS cell, autophagy.

1. Introduction

The production of induced pluripotent stem cells (iPSCs) is beneficial in regenerative medicine and provides new opportunities to understand the basic molecular mechanisms of human development and molecular aspects of degenerative diseases. Recent data suggest that iPSCs are influenced by the epigenetic memory (signature) of the tissue from which they are derived [1)]. For example, the DNA methylome, an epigenomic component, has a major contribution to phenotypic changes associated with aging [2, 3)]. Age-related epigenetic changes have been proposed to contribute to reduced physical and cardiac function, and accelerated epigenetic aging has been associated with disease and overall mortality in later life [2, 3)].

Autophagy maintains homeostasis in non-regenerative tissues by removing protein aggregates and cellular debris [4)]. However, this function is exacerbated with functional decline in cellular dysfunction with aging and inflammation [4)]. Disruption of autophagy leads to rapid accumulation of dysfunctional mitochondria, and diseases in which autophagy is impaired can result in severe cardiomyopathy [4)]. Therefore, autophagy and mitophagy pathways are promising new therapeutic targets for clinical treatment [4)]. However, the mechanisms by which cellular homeostasis is impaired and the effects of autophagy in restoring this function remain unclear.

Flavonoids are classified according to their chemical structure into flavones, isoflavones, flavonols, flavanonols,

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widely present in plants in the form of glycosylated or esterified glycosides [5,6]. Evidence is accumulating that flavonoids have anticancer effects, including autophagy, necrosis, inhibition of cell proliferation, cell cycle arrest, induction of apoptosis, cell migration, reduction of multidrug resistance in tumor cells, invasion, and tumor angiogenesis, and senescence[5,6]. Thus, flavonoids are a group of natural polyphenolic compounds characterized by multiple targets involved in multiple pathways and have been extensively studied in various models of autophagy regulation [7].

As described above, flavonoid-induced autophagy generally interacts with other mechanisms to influence its overall effect. In this study, we identified flavonoids from lemon and grapefruit, which have been implicated in promoting neuronal differentiation in a previous study [8], and examined the interaction of these flavonoids with autophagy and induction of GBA, a lysosomal enzyme, in iPS cells.

2. Materials and Methods

Chemicals and reagents:

Hesperidin (Synonyms: Hesperetin 7-rutinoside), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), dimethyl sulfoxide (DMSO), were obtained from WAKO.

Cell cultures:

hiPSC line was obtained from RIKEN Cell Bank (hiPS-RIKEN-1A). Undifferentiated hiPSCs were maintained on Matrigel (Corning)-coated dishes in ReproMed iPSC Medium (ReproCell) supplemented with 4 ng ml^{-1} human basic fibroblast growth factor (bFGF; WAKO). All cells were cultured at 37 °C in a humidified atmosphere of 3% CO_2 and 97% air.

Cell Treatment：

After reaching confluence, PSCs were treated with hesperidin (10 μM) and subfractions (10μg/ml) that were isolated from lemon and lemon peels.

Extraction and Isolation of citrus flavonoids

The lemon and grapefruit peels were extracted with ethanol, and the spectroscopic properties were measured. For the isolation of flavonoids from the fruit peels, ethanol extracts concentrated under reduced pressure at 45°C were fractionated over silica gel 60 eluted with solvent (n-buthanol: ethanol: water = 5:5:3 v/v) to give four subfractions. Column chromatography performed on silica gel 60 were monitored by Vis-UV spectrometer.

Preparation of crude enzyme solution:

The cells harvested by centrifuge (12,000 rpm for 3 min.) were frozen at -20℃ and thawed rapidly in distilled water, and was designated as the intracellular enzyme. The extracellular solution was dialyzed overnight against distilled water. The dialyzed solution was designated as the extracellular enzyme.

Bata-glucosidase activity:

Enzyme activities were measured by the activity against 0.25 % salicin. A 0.05-mL sample of an appropriately diluted enzyme solution was added to 0.05 mL of substrate in 0.1 M acetate buffer (pH 4.0), and the mixture was incubated at 30 °C. The reaction was stopped by the addition of a copper reagent, and the released reducing sugar was measured by the Somogyi-Nelson method [9].

MTT reagent-based cellular viability assay:

The MTT was used to quantitatively assess the viability of the iPS and cardiomyocyte cells. The cells were incubated with 10 % of MTT (37 °C; 24 h). After removing the supernatant, the insoluble formazan crystals were dissolved in 100 μL of acid-isopropyl alcohol. The absorbance in each well was measured at a wavelength of 570 nm, and regarded as cellular viability.

3. Results and Discussion

3. 1 Spectroscopic analysis of citrus extracts

Citrus fruits are often characterized by their bright color, sweet or sour taste, nutrients such as vitamin C, potassium and folate, and the presence of the flavonoids such as hesperidin and naringin. The flavonoid naringin is found in citrus fruits, especially grapefruit, and hesperidin is most abundant throughout citrus fruits such as oranges, limes, and lemons, although the amount varies among fruit types[10]. Accordingly, compounds isolated from grapefruit and lemon show absorbance around 284 or 275, indicating that they could be naringin and hesperidin, respectively (Fig. 1).

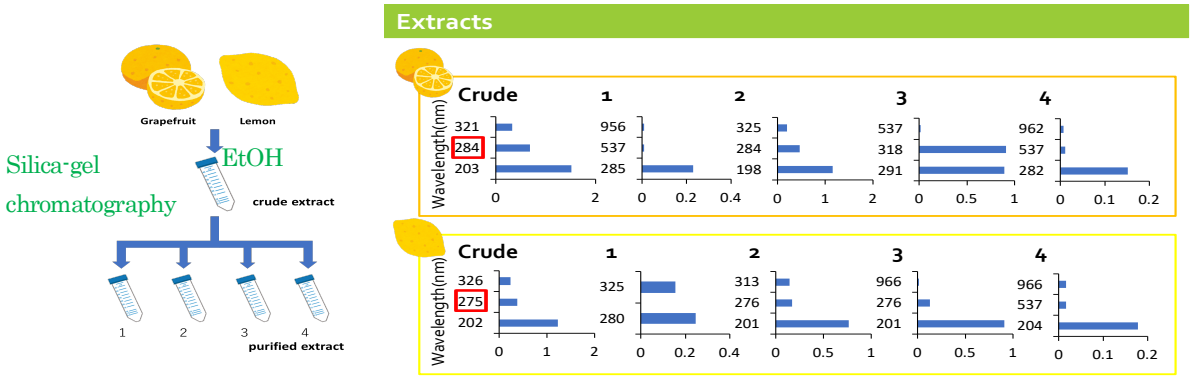

Figure 1 Purification and identification of polyphenol from the grapefruit and lemon extract. Spectroscopic analysis of the crude extract and the purified extracts. The extract number shown in the figure indicates fractions of grapefruit and lemon extracts with silica-gel chromatography.

3. 2 Proliferative effect of grapefruit and lemon extracts on iPS cells

Proliferation of iPS cells increased with treatment of grapefruit-derived subfraction 1 and decreased with treatment of lemon-derived subfraction 2 (Fig. 2). Flavonoids have certain apoptosis inhibitory effects and are capable of reducing the area of myocardial infarction significantly. The anti-apoptotic effect on hydroxyl groups on the carbon chain and its intensity on the existence of hydroxyl groups on the A and C rings weaken its anti-apoptotic effect, since the hydroxyl groups of phenol are stronger than those of alcohol-hydroxide, the hydroxyl groups on the B ring. This negative effect, however, is counterbalanced by the hydroxyl group on the A ring. The mechanism may involve the suppression of expression of pro-apoptotic genes (e.g., Bax) and the promotion of expression of anti-apoptotic genes (e.g., Bcl-2). Interactions between some signaling molecules in the apoptotic signaling pathway are also beneficial in suppressing the occurrence of apoptosis[11]. Naringin has two hydroxyl groups on the A ring and one on the B ring, while hesperidin has one hydroxyl group on the A ring and one on the B ring. Therefore, naringin included in grapefruit is theoretically more effective in preventing apoptosis, thereby supporting the results. Naringin, a flavone glycoside, is inexpensive and widely available, and its long history of use and diverse pharmacological actions make it promising for the treatment of many diseases, with evidence currently focused on its anticancer effects. Naringin has been demonstrated to employ multiple mechanisms to initiate, promote, and impede cancer progression by modulating several aberrant signaling cascades involved in inflammation, proliferation, cell survival, apoptosis, autophagy, angiogenesis, invasion, and metastasis [12]. Our findings suggest that naringin inhibits apoptosis and prevents cell death of iPS cells. (Fig. 2). Hesperidin (HES), on the other hand, has many pharmacological activities, including anti-inflammatory, antioxidant, and promoting osteoblast[13] and neuronal cell differentiation[14]. Normally, the

proliferation rate of cells decreases as they differentiate, and most cells in adult animals are arrested in the G0 phase of the cell cycle[15]. The present results suggest that hesperidin induces iPS cell differentiation and a decrease in cell proliferation.

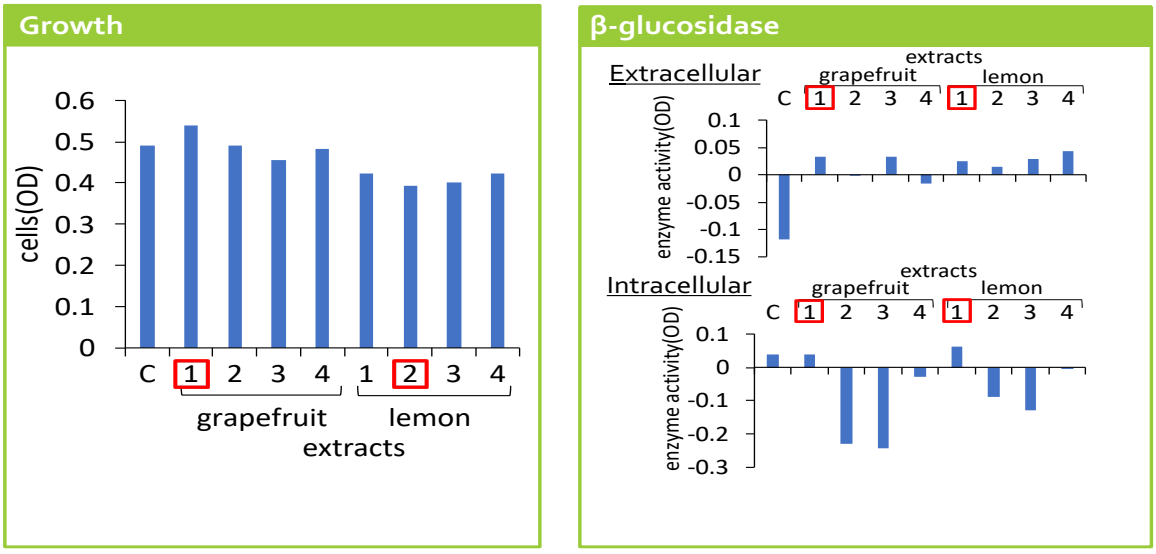

Figure 2 Cell Proliferation and the β-glucosidase activities of iPS cells treated by grapefruit and lemon extracts.
The grapefruit and lemon extracts were added to iPS-cell cultures and their number of cells and β-glucosidase activities were measured. The extract numbers; see figure 1.

3. 3 The β-glucosidase activities of iPS cells treated by grapefruit and lemon extracts

Beta-glucosidase is a lysosomal glycosidic hydrolytic enzyme that catalyzes the hydrolysis of aryl and alkyl-ß-D-glucosides, as well as of glucosides with only the sugar chain portion. Loss of this enzyme leads to lysosomal storage disease, in which lipid derived products and abnormal proteins accumulate. Treatment of grapefruit and lemon subfraction 1 increased extracellular and intracellular ß-glucosidase activity (Fig. 2). Hesperidin and naringin are flavanone glycosides characterized by the presence of saturated C2-C3 bonds in the C ring and usually present as a racemic mixture. Flavanone glycosides have beneficial effects such as inhibition of the development of neurodegenerative diseases, anti-inflammatory effects, and antioxidant effects. Various anticancer effects have also been reported for many flavonoid glycosides. However, although the putative mechanisms of action of flavonoids as drugs are diverse and the role of glycosylation is often not well understood[16], pharmacological chaperone (PC) therapy has been proposed and studied as a treatment for many genetic diseases caused by misfolded or unstable proteins, including LSD　Small molecule PCs are designed to selectively bind to and stabilize mutant proteins to promote proper folding and intracellular trafficking, thereby increasing the total amount and activity of intracellular enzymes[17]. Human cytosolic ß-glucosidase shows significant activity and relatively high affinity and specificity for several plant-derived ß-D-glucosides of flavones, isoflavones, and flavonols[18]. Thus, ß-D-glucoside may bind as a substrate and stabilize ß-glucosidase as a molecular chaperone, which may explain the high enzyme activity maintained.

3. 4 The grapefruit and lemon extracts modulate autophagy activity

Less glycosylation was observed in the iPS cell preparations with grapefruit fraction 1 and lemon fraction 2 (Fig. 3; Glycosylation), while protease activity was increased in the preparations with grapefruit fractions 3 and 4 and lemon fractions 1 and 2 (Fig. 3; Proteinase). These results indicate that glycoproteins are transported to lysosomes for autophagic catabolism and potentially degraded by a combination of proteases and glycosidases. The catabolic pathway for high mannose, hybrid, and complex-type glycans is bidirectional, with removal of monosaccharides from the nonreducing termini by exoglycosidases, proteolysis, and digestion of glycan-polypeptide bonds proceeding simultaneously. Most lysosomal glycosidases are thought to be involved in the degradation of O-linked glycans. Because the enzymes act on the same linkages regardless of whether they are N-linked glycans, O-linked glycans, glycosaminoglycans, or glycolipids. Defects in these pathways then cause lysosomal storage disease. Improperly folded or glycosylated proteins are degraded in the ER and cytoplasm, and the end products of cytoplasmic degradation of N-glycans are delivered to lysosomes. The pathway becomes enhanced in cells that are highly secretory of glycoproteins or have increased production of abnormal glycoproteins. Thus, the interaction between lysosomes and proteasomes is important for the maintenance of cell function[19].

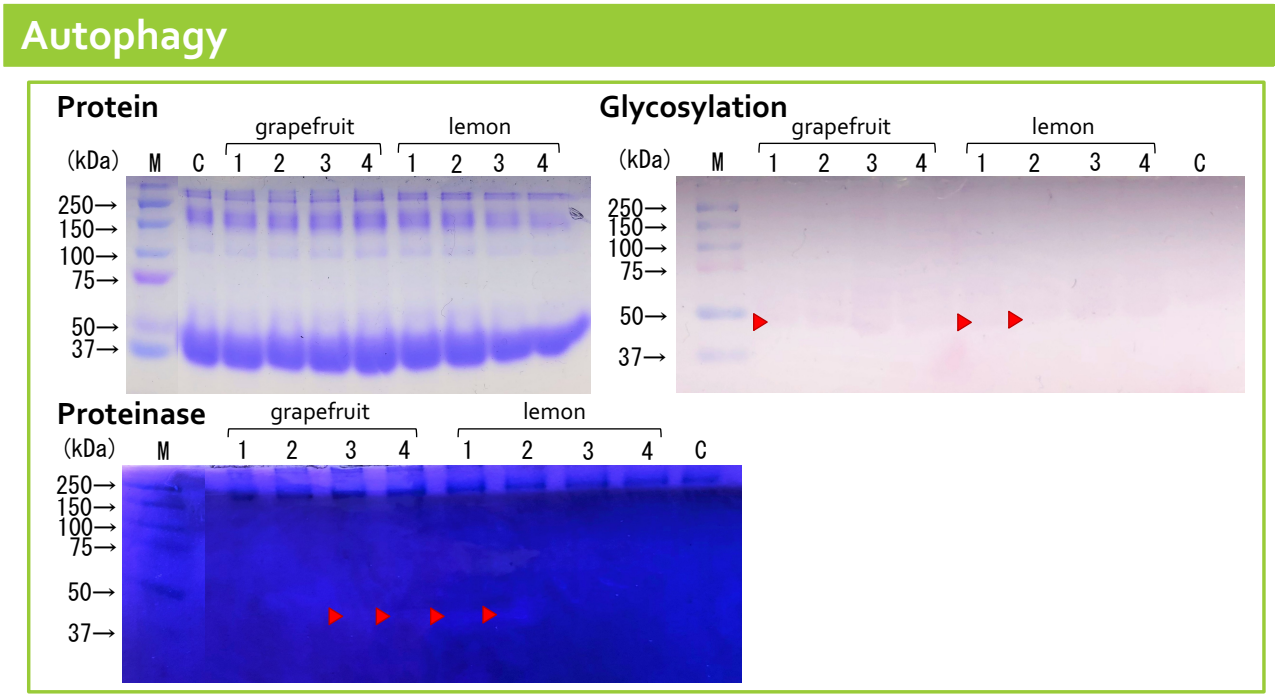

Figure 3 The grapefruit and lemon extracts modulate autophagy activity. The SDS-PAGE for proteins from the iPS cells (A). The indexes of the autophagy activity are glycosylation of proteins (B) and proteinase activity(C) of iPS cells. The extract numbers; see figure 1.

3. 5 Receptor activation of iPS cells treated by the grapefruit and lemon extracts

The expression levels of LC3 Ⅱ and GBA were significantly elevated in the lemon fraction (Fig. 2, 4). LC3 is an important and reliable marker of autophagy; LC3 exists in cytoplasmic (LC3I) and membrane-bound (LC3II) forms; an increase in LC3II is thought to be closely related to the extent of autophagosome formation [20]. Hesperidin has been reported to both inhibit and promote autophagy. Excessive autophagy in ischemia/reperfusion-induced myocardium is suppressed by hesperidin, which activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway[21].On the other hand, in experimental colon carcinogenesis model and non-small cell lung cancer cell lines, apoptosis and autophagy are promoted by suppressing Aurora kinase via the PI3K/AKT and mTOR pathways and by the

FGF and NF-κB pathways [22]. Lemon fraction 1 increased LC3II and ß-glucosidase, suggesting that hesperidin in lemon fraction 1 activates autophagy, and the activation of autophagy may be a pathway mediated by downregulation of estrogen receptors.

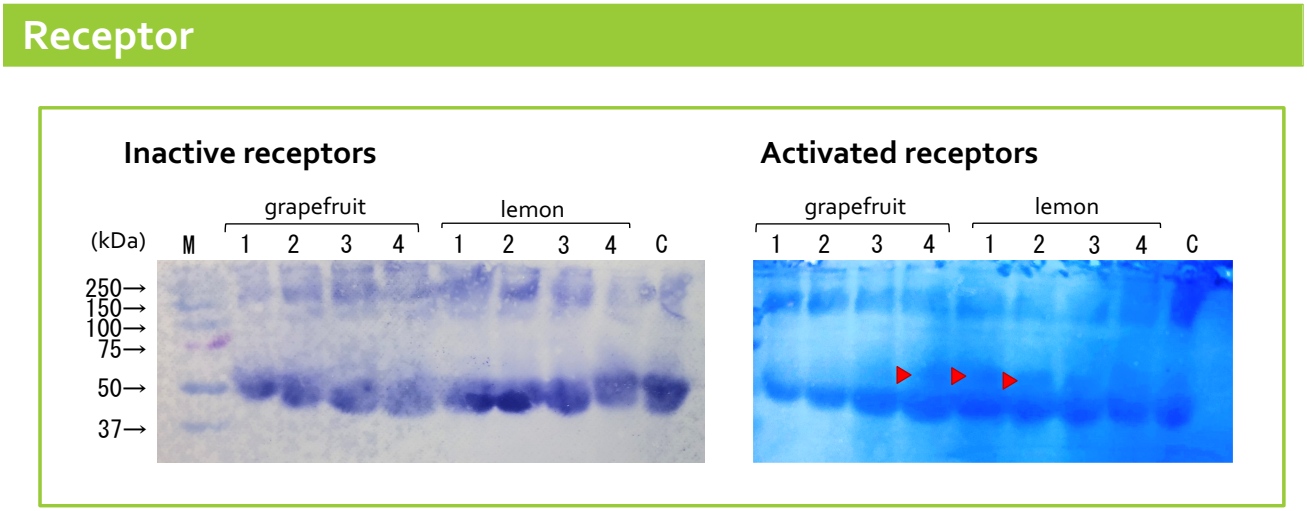

Figure 4 Receptor activation of iPS cells treated by the grapefruit and lemon extracts. The proteins fractionated by semi SDS-PAGE were transferred onto PVDF (inactive receptors) and nitrocellulose (activated receptor) membranes. The extract numbers; see figure 1.

3. 6 Proliferative effect of Hesperidin and lemon extracts on iPS cells

Since the fraction 1 from the lemon extract was thought to be hesperidin, the cell proliferation and the intracellular β-glucosidase activity were examined on hesperidin. Similar cell proliferation rates and β-glucosidase activity were observed in cultures with hesperidin and lemon fraction 1, supporting the spectroscopic analysis that lemon fraction 1 has hesperidin.

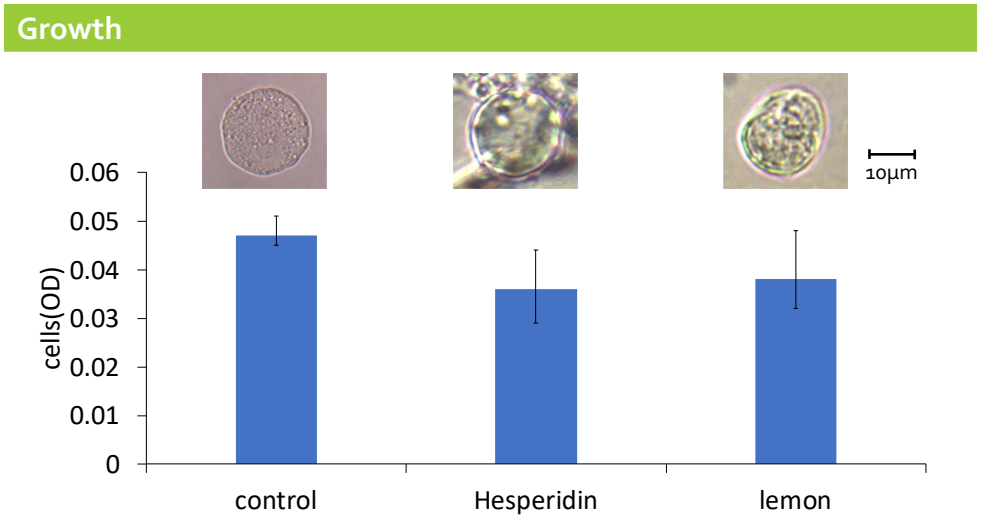

Figure 5 Proliferative effect of hesperidin and lemon extracts on iPS cells. Hesperidin and lemon extracts were added to iPS-cell cultures and their number of cell were measured (bottom). The fraction 1 of lemon extracts was purified with silica-gel chromatography. Representative images of β-glucosidase activities in iPS cells treated by hesperidin and the fraction 1 of lemon extracts (top). Each solution was added to iPS-cell culture and the cells were observed under microscope. Scale bars, 10μm.

4. Conclusion

The effects of lemon and grapefruit extracts on autophagy of iPS cells were investigated, revealing that hesperidin contained in lemons activates autophagy of iPS cells. The activation of autophagy by hesperidin depends on the cell type, namely, autophagy is inhibited in cardiomyocytes to prevent cell damage, while autophagy is activated in cancer cells to promote apoptosis. Activation of autophagy by hesperidin indicates that hesperidin activates autophagy via inhibition of mTOR. The absence of severe cell loss associated with apoptosis in iPS cells treated with hesperidin suggests that the activation of autophagy promotes cell differentiation, resulting in a reduced cell proliferation rate. The transforming growth factor-β (TGF-β) superfamily is a type of estrogen receptor that regulates cell differentiation. The TGF-β directs the fate of pluripotent stem cells (in the neural crest) by modulating the expression and function of tissue-specific transcription factors and by regulating the expression of necessary growth factors and their receptors selectively (in the mesenchyme). During skeletogenesis, TGF-β acts to regulate the differentiation of chondrocytes and osteoblasts; responsiveness to TGF-β varies as cells differentiate, and evidence now indicates that changes in TGF-β receptor profiles may explain part of these differences [23]. Thus, hesperidin, a lemon flavonoid, could stimulate autophagy activity in iPS cells through the TGF signaling pathway, a cytokine that blocks growth signals. In addition, lysosomes activated by hesperidin could promote metabolic shifts that mobilize glycoproteins for degradation and reset proteostasis by wrapping and clearing glycoproteins. Therefore, the lysosomal switch that enhances proteostasis of iPS cells toward differentiation induced by hesperidin may be beneficial for the maintenance of iPS cell function.

5. Acknowledgments

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