Thyroid-stimulating hormone stimulation downregulates autophagy and promotes apoptosis in chondrocytes

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Abstract. Subclinical hypothyroidism (SCH) patients have normal thyroid hormone levels but increased thyroid stimulating hormone (TSH) level in serum. It has been reported that high TSH is related to abnormal skeletal development in mice with hypothyroidism. However, the cellular mechanism is not fully understood. In the present study, we aim to investigate the direct effects of TSH stimulation on chondrocytes, and the putative role of autophagy in this process. By using EdU incorporation assay and flow cytometry for mitochondrial membrane potential assay, we demonstrated deceased proliferation and promoted apoptosis in TSH stimulated primary mouse chondrocytes. And the balance of Bcl-2 and BAX expression on protein level was broken. More interestingly, the expression of autophagic markers Beclin-1 and LC3II was reduced in TSH stimulated chondrocytes, accompanied by less autophagosomes and accumulated p62 protein, indicating an impaired autophagic flux. More interestingly, mTOR was upregulated and AMPK activity was decreased in TSH stimulated PMCs, suggesting that mTOR/AMPK pathway is get involved in the regulation of TSH on autophagy in PMCs. Collectively, we found an increased apoptosis and suppressed autophagy in TSH stimulated primary chondrocytes, which is meaningful in understanding the effects of increased TSH level on articular cartilage and the role of autophagy in this process, and thus provide a potential novel therapeutic target in related cartilage damages.

Key words: TSH, Chondrocyte, Proliferation, Apoptosis, Autophagy

OSTEOARTHRITIS (OA) is the most common form of arthritis that affects about 3.8 % of people in the world [1]. OA results from the breakdown of articular cartilage, which is an avascular tissue with limited regenerative ability. As the only cell type in cartilage, the chondrocyte death / survival has a key role in cartilage maintenance and functionality. Apoptosis in chondrocytes has been detected in articular cartilage derived from OA patients [2], suggesting a crucial role for chondrocyte apoptosis in the pathogenesis of OA. However the mechanism is not fully understood.

Thyroid-stimulating hormone (TSH) is a glycoprotein hormone. It mainly regulates the synthesis and secretion of thyroid hormone by interacting with TSH receptor (TSHR) on thyroid follicular cells. Other putative roles of TSHR have been reveals in recent years with the facts that TSHRs are expressed in many extra-thyroidal tissues, such as adipose tissue, bone and liver [3-5]. TSH has been identified to have direct effects on these tissues independently of thyroid hormone [6-8]. In 2013, Endo *et al*. reported that TSHR was highly expressed in cartilage tissue at levels comparable to that seen in the thyroid [9]. However the effects of TSH on chondrocyte behavior remain unclear.

Autophagy is a highly conserved degradation process involved in the clearance of damaged intracellular proteins and organelles [10]. To maintain cell homeostasis under various stress conditions, autophagy has been related to the apoptosis in many cell types, such

as retinal cells [11], proximal tubular cells [12] and hepatocytes [13]. In this study, we aim to investigate whether TSH has direct effects on chondrocyte apoptosis and the involvement of autophagy in this process.

Materials and Methods

Cell culture and treatment

The primary mouse chondrocytes (PMCs) were isolated from the epiphyseal cartilage of newborn C57BL/6 mice as described earlier [14]. The culture medium was supplemented with 44 mg/ml ascorbic acid (Sigma-Aldrich, USA) and 130 mg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, USA) to ensure the collagen hydroxylation. The cells were stimulated with 10 mU/ml TSH from bovine pituitary (Sigma-Aldrich, USA) for 48 hours before harvest.

EdU assay for cell proliferation

Cells were seeded at a density of 2×10^4 cells/well in a 96-well plate under standard conditions and treated with 10mU/ml TSH. The proliferation of PMCs was detected by DNA incorporation using 5-ethynyl-2'-deoxyuridine (EdU) kit (RiboBio, China) according to the manufacturer's instructions. Images were acquired by immunofluorescence microscopy (Nikon Ti-S, Tokyo, Japan).

Determination of mitochondrial membrane potential for cell apoptosis

Cells were seeded on 6-well plates. After 48 h incubation, cells were digested with 0.1 % collagenase P and collected by centrifugation. The Mitochondrial Membrane Potential Detection Kit (BD, Germany) was used to analyze cell apoptosis according to the manufacturer's instructions with flow cytometry analysis (BD LSRFortessa). In brief, color from red fluorescence to green fluorescence of JC-1 indicated a change in the mitochondrial membrane potential.

Western blot analysis

The procedure of western blot analysis was performed as previously described [15]. The following primary antibodies were used: mouse anti-SQSTM1/p62 (Abcam, ab56416), rabbit anti-LC3B (Abcam, ab48394), rabbit anti-Beclin-1 (Santa Cruz, SC-11427), rabbit anti-mTOR (Abcam, ab2732), rabbit anti-phospho-AMPKα (CST, #2535), rabbit anti-AMPKα (CST, #2532). Primary antibody against β-actin and horserad-

ish peroxidase-conjugated secondary antibodies were from ZSGB-BIO.

Immunofluorescent staining

After treatment, cells were fixed in 4 % paraformaldehyde for 15 min at room temperature, then washed and permeabilized using PBS containing 0.3 % Triton X-100, followed by blocking with 1 % NGS in PBS. Primary antibodies (1:100) were added to the cells. After incubating overnight at 4 °C, cells were washed 3 times with TBST. Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies (1:1,000) were added and incubated for 1h. Finally, Diamidino-phenyl-indole (DAPI) was used to label the nuclei. Images were acquired by immunofluorescence microscopy (Nikon Ti-S, Tokyo, Japan). p62 immunofluorescent staining and the apoptotic nuclei stained by Hochest 33258 (Thermofisher, USA) were visualized by a scanning confocal microscope (Carl Zeiss, LSM 780).

Electron microscopy

After treatments, the PMCs were scraped gently and collected into centrifuge tubes after washing by PBS. Then, the cells were fixed in 3 % glutaraldehyde at 4 °C, dehydrated by graded ethanol rinses before dimethylketone. After embedded in Epon-812, the samples were cut into ultrathin sections (70 nm). Uranium acetate and plumbumcitrate dyed the ultrathin sections. They were observed with JEOL-1200EX electron microscopy.

Statistical analysis

Results are presented as means ± SD. Data were analyzed using SPSS17.0 software. Significance was assessed using unpaired Student's t test. Three independent experiments were performed and $p < 0.05$ was considered significant.

Ethics statement

The animal study was approved by the institutional review board of Shandong provincial hospital affiliated to Shandong University (No. 2013-067).

Results

TSH reduced the proliferation of PMCs

To investigate the effect of TSH on chondrocyte proliferation, primary mouse chondrocytes were isolated and stimulated by 10 mU/ml TSH. Then the cell

proliferation was analyzed by EdU incorporation assay. As shown in Fig. 1, TSH dramatically reduced the EdU incorporation into DNA in PMCs by 6 fold of that of non-treated control cells.

TSH induced the apoptosis of PMCs via BAX / Bcl-2 pathway

The chondrocyte apoptosis was then analyzed by measuring mitochondrial membrane potential. As shown in Fig. 2A, TSH stimulation increased the depolarized mitochondrial membrane potential, which was indicated by the increase of the lowered red fluorescence signal in R2 channel. This apoptotic population was increased about 2.5 fold after stimulated by TSH (Fig. 2B). We further showed that apoptosis inducer BAX protein expression was increased while Bcl-2 was decreased upon TSH stimulation (Fig. 2C, D). Thus the ratio of Bcl-2 and BAX protein level was reduced in TSH treated cells, indicating that TSH induces

apoptosis in PMCs, which may be dependent on the BAX / Bcl-2 pathway.

TSH decreased the autophagy level in PMCs

To investigate the role of autophagy in TSH induced apoptosis in PMCs, autophagy was analyzed by measuring autophagic markers LC3 and Beclin-1 protein levels. First western blotting showed that both LC3II and Beclin-1 expression were decreased upon TSH stimulation compared to non-treated control cells (Fig. 3A, B). This was further confirmed by immunofluorescent staining (Fig. 3C).

TSH reduced autophagosomes in PMCs

Autophagosomes were visualized by transmission electron microscopy (TEM) in cultured PMCs. There were significantly less autophagosomes in TSH treated PMCs compared to non-treated control cells (Fig. 4), indicating an impaired degradation capacity.
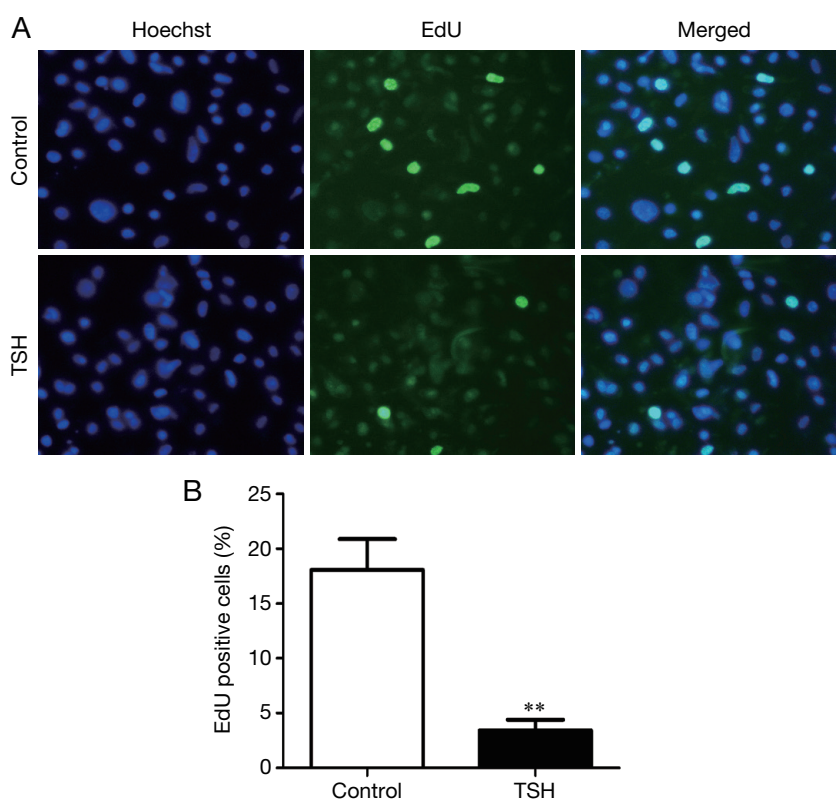

Fig. 1 Decreased proliferation in TSH stimulated PMCs

A) EdU positive cells (green) were reduced in PMCs treated with 10 mU/ml TSH for 48 h compared with the control cells. Nuclei were identified by hoechst staining. **B**) Quantification of EdU positive cells indicated that the significant reduction of cell proliferative capacity after TSH treatment. Values are mean ± SD, n = 3, ** $p < 0.01$.
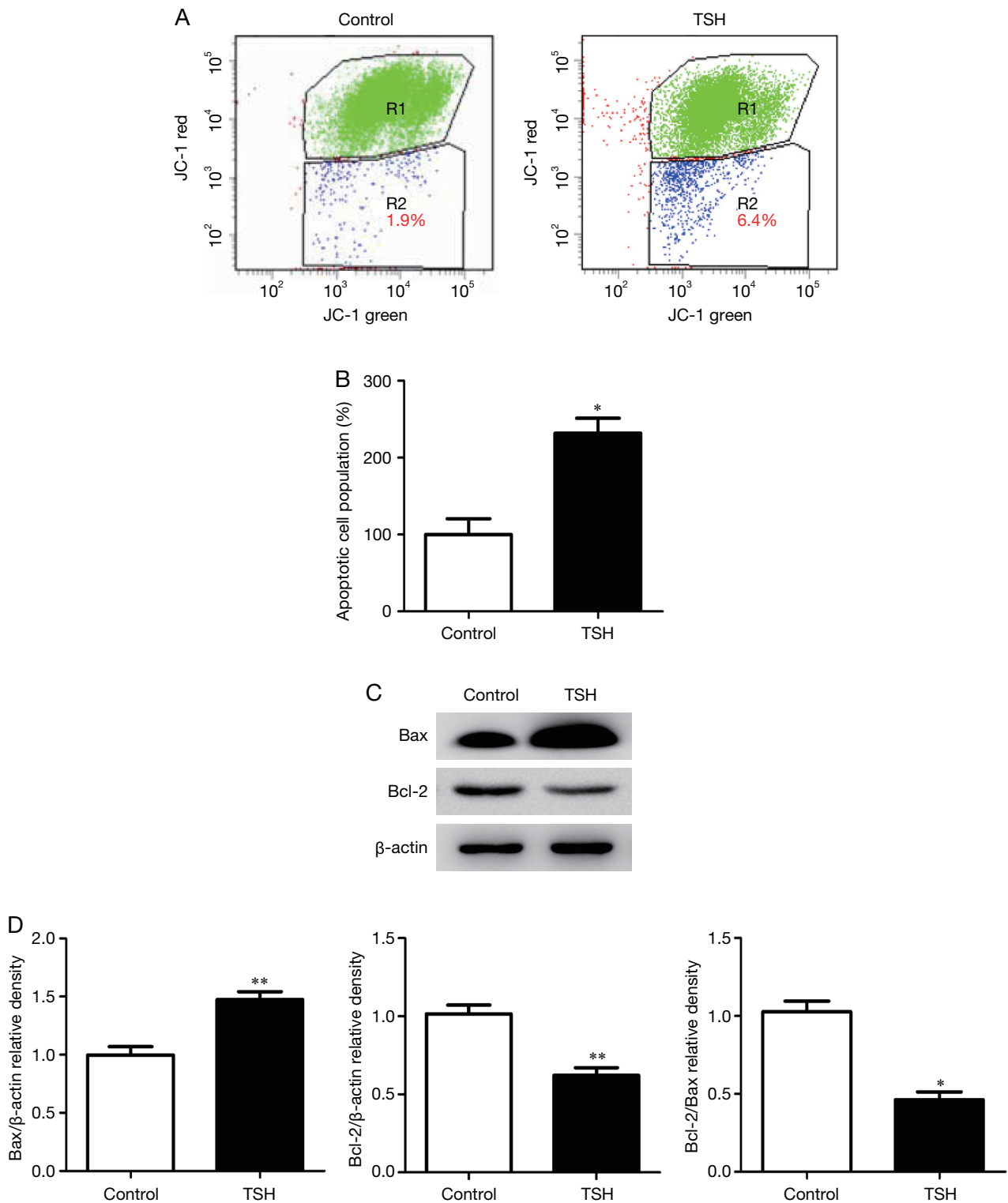
Fig. 2   Increased apoptosis in TSH stimulated PMCs
   A) The mitochondrial membrane potential of cells was evaluated by flow cytometry.   **B**) Apoptotic cell populations of lowered red fluorescence representing were calculated.   **C**) Western blotting for Bax and Bcl-2 in PMC cells treated with or without 10 mU/ml TSH for 48 h.   **D**) Relative density of Bax and Bcl-2 and their ratio were quantified.   * $p < 0.05$, ** $p < 0.01$.
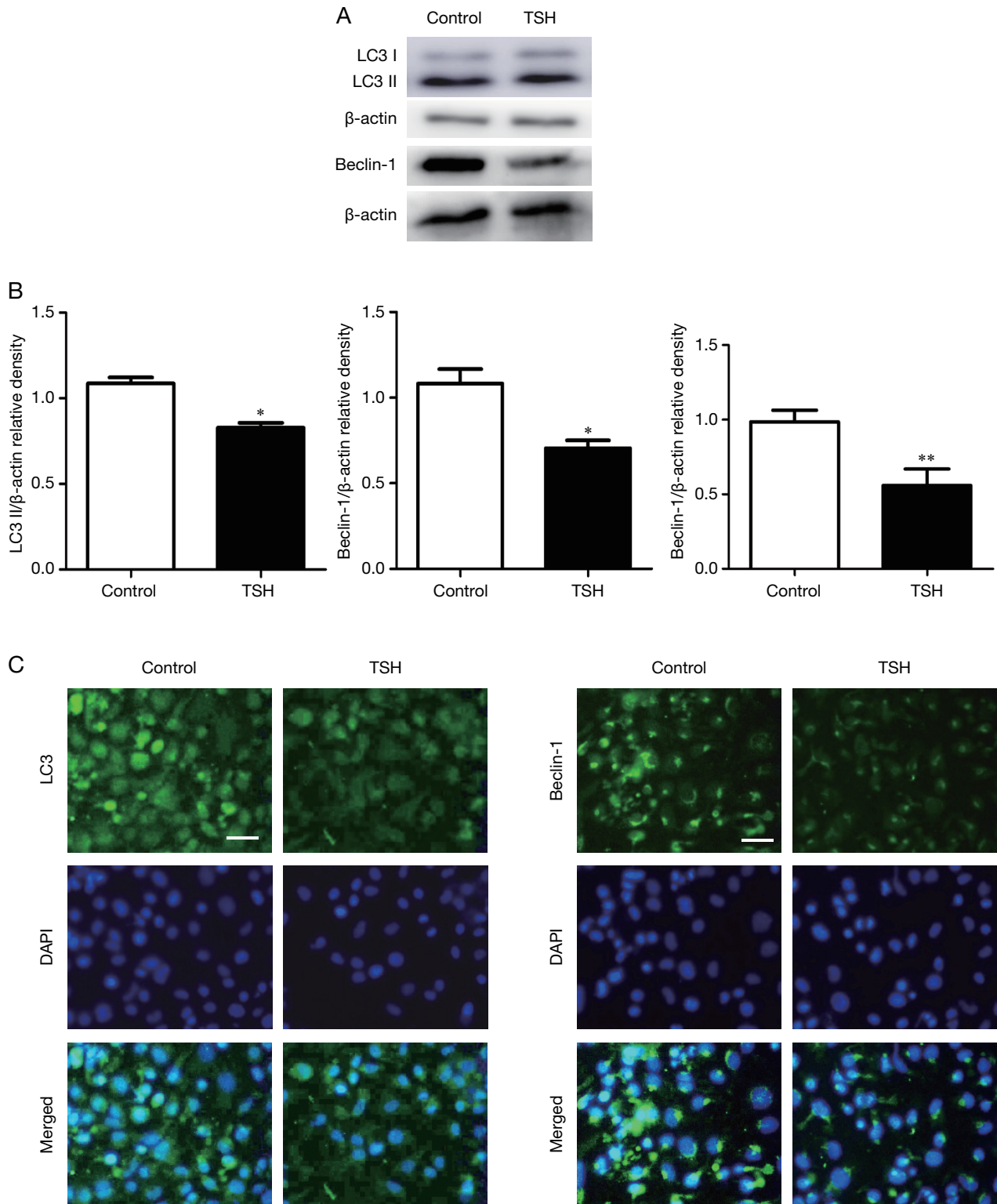
Fig. 3 Decreased protein levels for autophagic markers

A) Western blotting for LC3 and Beclin-1 in cell lysates treated with or without 10 mU/ml TSH for 2 h. **B**) Relative density of A. * $p < 0.05$. **C**) Immunostaining for LC3 and Beclin-1 in cells treated with or without 10 mU/ml TSH for 2 h. Bar = 40 μm.
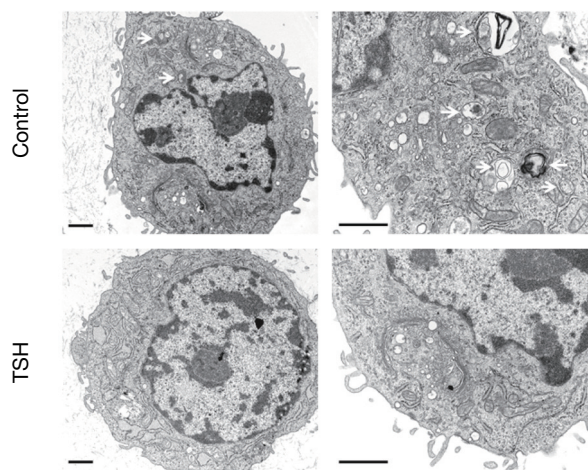
Fig. 4   Electronic microscopy analysis of PMCs treated with or
without 10 mU/ml TSH for 2 h
White arrows indicate autophagosomes. Bar = 1 μm.

TSH impaired autophagy degradation in PMCs

The protein p62, also known as sequestosome 1, is
normally used as a marker of autophagic degradation,
as it localizes to autophagosomes via the interaction
with LC3 and to be constantly degraded by the autoph-
agy-lysosome system [16]. As shown in Fig. 5A,
expression of p62 was increased significantly in TSH
stimulated PMCs, and its accumulation was further
confirmed by immunostaining (Fig. 5C). Furthermore,
some of the cells expressing higher p62 were co-local-
ized with condensed or fragmented nuclei stained
with Hoechst 33258 as shown in white arrows in Fig.
5C, indicating the role of impairment of autophagy in
inducing apoptosis in TSH stimulated PMCs.

Impaired mTOR/AMPK pathway in TSH stimulated PMCs

The upstream autophagy regulators mTOR and
AMPK were then analyzed by western blotting after
PMCs were treated with or without TSH. As shown in
Fig. 6, the expression of mTOR was increased in TSH
stimulated cells. However, the relative phospho-AMPK
amount was dramatically decreased compared to the
total AMPK.

Discussion

Subclinical hypothyroidism (SCH) is a common
and frequently occurring thyroid disease with nor-
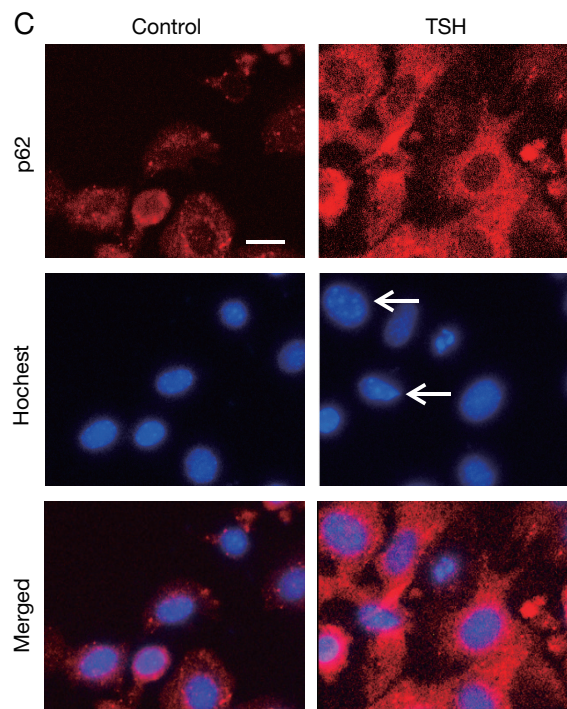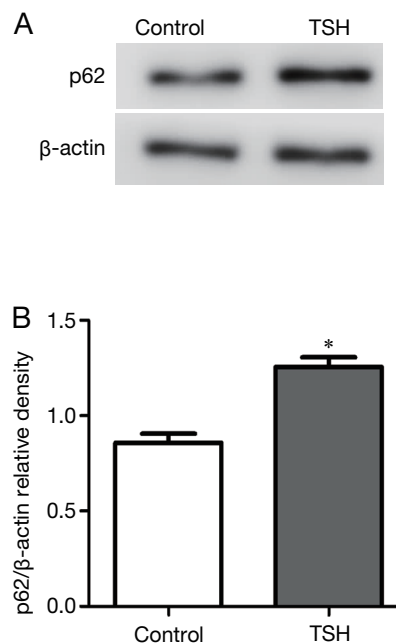mal thyroid hormone levels and increased TSH level

Fig. 5   Increased accumulation of p62 protein
A) Western blotting for p62 in PMCs treated with or
without 10 mU/ml TSH for 2 h. **B**) Relative density of A.
* $p < 0.05$. **C**) Immunostaining for p62 in PMCs treated
with or without 10 mU/ml TSH for 2 h. The nuclei were
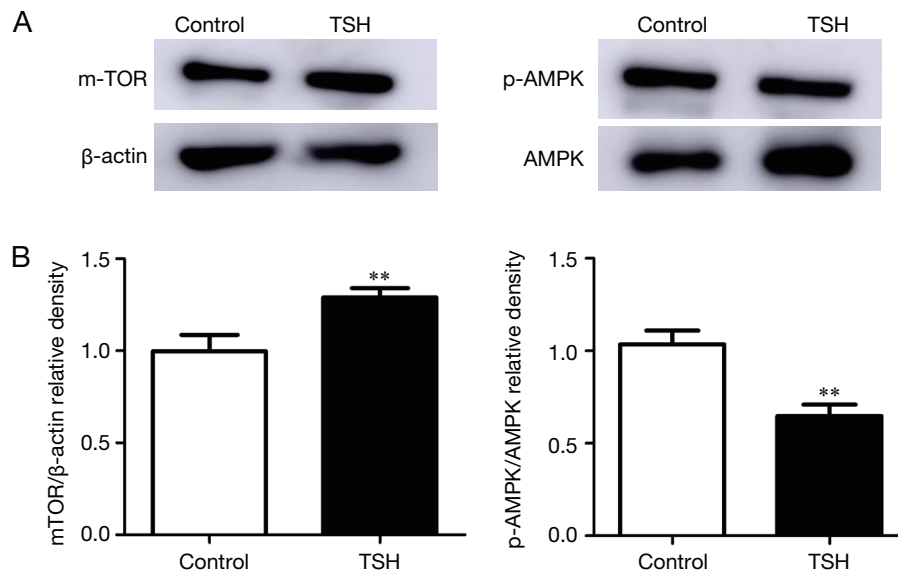counterstained by Hochest 33258. Bar = 25 μm.

Fig. 6 Altered mTOR/AMPK pathway after TSH stimulation

A) Western blotting for AMPK, pAMPK and mTOR in cell lysates treated with or without 10 mU/ml TSH for 48 h. **B)** Relative density of A. * $p < 0.05$.

in serum. In recent years, emerging data show that TSH has a direct function on some non-thyroid tissues independent of thyroid hormones. However, the role of TSH on non-thyroid tissues is under debate. It was reported that TSH stimulated the proliferation of vascular smooth muscle cells [17] and have a direct role in the progression of atherosclerosis by regulating blood sugar and lipid metabolism [17-19]. However, TSH is a negative regulator in bone remodeling by inhibiting osteoclast mediated bone resorption [20, 21]. In the present study, our first finding is that TSH has a negative effect on the proliferation of PMCs, and thus gave a direct evidence for the relationship between the pituitary axis and the skeletal system. This is consistent with the study of Endo *et al*. that high serum TSH contributes to the growth retardation in the affected animals by comparing two distinct mouse models of hypothyroidism with or without a functional TSHR [19]. Our study provides the direct cellular basis underlying the phenotype they observed.

We first established the fact that TSH reduced the proliferation of PMCs and increased their apoptosis (Figs. 1, 2). As the only cell type in the cartilage, the survival of chondrocytes is vital for the maintenance of healthy cartilage. And chondrocyte apoptosis plays an important role in cartilage degeneration [22]. Thus we speculate that the stimulation of TSH would increase

the susceptibility of OA in articular cartilage. And in clinic the susceptibility and severity of OA in SCH patients should be paid more attention.

Mitochondria are the important sites and key regulators for cell apoptosis. The mitochondrial anti-apoptotic protein Bcl-2 inhibits the release of cytochrome c (cyt-c) from mitochondria, whereas BAX promotes its release and the loss of mitochondrial membrane potential, which eventually leads to the cellular apoptosis. In the present study, the stimulation of TSH in PMCs broke the balance between these proteins as we detected the decreased expression of Bcl-2 and increased expression of BAX. And the mitochondrial membrane potential was also altered (Fig. 2). Thus the chondrocyte apoptosis induced by TSH is Bcl-2 signaling mediated.

Autophagy is an evolutionary conserved self-eating process and is a protective mechanism against cellular stresses. Autophagy failure has been related to various human diseases such as neurodegeneration, cardiomyopathies and abnormal skeletal development [23, 24]. Recently it has been suggested that autophagy is dysregulated during OA [25]. The present study demonstrated that the expression of LC3II and Beclin-1 was decreased in TSH stimulated epiphyseal chondrocytes (Fig. 3), revealing autophagy was suppressed under this circumstance. This is consistent with the

finding of Caramés *et al*. that in human OA articular cartilage, the expression of autophagic markers including Beclin-1 and LC3 was reduced, which was accompanied by an increase in apoptosis [26]. In addition, condensed or fragmented nuclei in TSH stimulated PMCs were partially co-localized with increased p62 staining. Thus we speculate that the suppressed autophagy by TSH is at least partially responsible for the increased cellular apoptosis, which needs to be evaluated in future work.

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase, and plays a vital role in regulating cell metabolism, growth and survival. There are evidences showing that mTOR and AMP activated protein kinase (AMPK), a sensor of cellular energy status, coordinate the mammalian autophagy initiation by directly regulating Ulk1 [27, 28], and that AMPK promotes autophagy by directly phosphorylating Ulk1, while mTOR prevents Ulk1 activation by disrupting the interaction between Ulk1 and AMPK and thus inhibits autophagy. Based on these studies, we hypothesized that mTOR/AMPK pathway may be involved in the regulation of TSH on autophagy in PMCs. In the present study, we could show that mTOR was high expressed in TSH stimulated PMCs. In parallel, the AMPK activity was suppressed, revealed by the decreased phosphorylated AMPK amount (Fig. 6). This finding is coordinated with Andrade's study that TSH could suppress AMPK activation in the thyroid gland [29]. On the other hand, Zhang *et al*. found that mTOR was overexpressed in human OA cartilage and cartilage-specific deletion of mTOR upregulates autophagy in OA mice. In addition, the upregulation of mTOR expression co-related with increased chondrocyte apoptosis and reduced expression of key autophagy genes during OA [30]. Similarly, in our study, we found increased apoptosis and upregulated mTOR expression; as well as reduced autophagy level in TSH stimulated PMCs. It will be very interesting to monitor if inhibition of mTOR can also protect PMCs from TSH induced apoptosis in future work.

In addition, we also found reduced autophagosomes and accumulated p62 in TSH stimulated PMCs (Figs. 4, 5), indicating an impaired autophagic flux. Emerging studies have revealed that the failed degradation of damaged organelles and unnecessary proteins, as well as the accumulated aggregates are harmful to the cells and play important roles in many pathologic conditions [31, 32]. This could also be a potential mechanism for TSH induced apoptosis in PMCs.

In conclusion, our study demonstrated an increased apoptosis in TSH stimulated PMCs, which is Bcl-2 signaling mediated. The expression of autophagic markers Beclin-1 and LC3II was reduced, accompanied by a suppressed autophagy flux with accumulated p62 protein. Our findings are meaningful in understanding the effects of increased TSH level on articular cartilage and the role of autophagy in this process. And the restoration of autophagy may be a novel therapeutic method in related cartilage damage.

Acknowledgements

Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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