

The Effects of sKlotho on Osteoblast Metabolisms under High Glucose and its mechanisms

Xiaoyu Ma

The First Hospital of China Medical University

Can Wu

The First Hospital of China Medical University

Fenqin Chen

The First Hospital of China Medical University

Xiujuan Lv

Fourth People's Hospital of Shenyang

Qiuyue Wang (✉ wqycmu123@163.com)

The First Hospital of China Medical University

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Abstract

Background Studies have found that excessive reactive oxygen(ROS) productions could cause senile osteoporosis and diabetic osteoporosis(DOP). Silent information regulator 1 (Sirt1) has anti-oxidative and anti-aging effects on improving bone metabolism through P53/P21 and P16 pathways in senile osteoporosis. Klotho is a kind of anti-aging gene and Klotho-/- mice is characterized by osteoporosis. The secreted Klotho(sKlotho) proteins exert anti-oxidative stress effect in multiple systems. Our study is aimed to explore the effects of sKlotho on osteoblast metabolisms, Sirt1 expression and senescence pathway under high glucose. Results We found that sKlotho could improve MC3T3-E1 proliferation and differentiation, reduce cell apoptosis and ROS, increased Sirt1 expression, and inhibit P53/P21 and P16 activation under high glucose. While the improvement of MC3T3-E1 activity and anti-aging effect of sKlotho on MC3T3-E1 under high glucose disappeared after transfected with Sirt1-siRNA. Conclusions We concluded that sKlotho could significantly improve the activity of MC3T3-E1, alleviate oxidative stress and senescence under high glucose via increasing the expression of Sirt1 by reducing ROS production.

Background

Diabetic osteoporosis(DOP) is a systemic metabolic bone disease characterized by diabetes mellitus(DM) complicated with osteopenia, change of bone microstructure, bone strength decreased and fragility increased which is easy to cause fracture. Studies have showed that oxidative stress(OS) is an important cause. Excessive oxygen species(ROS) and inflammatory factors could cause bone metabolic homeostasis disbalance and damage bone matrix directly by regulating multiple cell signaling pathways in DOP[1,2].

Silent information regulator-2(Sir2) is a kind of type III histone deacetylation enzyme which has highly conserved nicotinamide adenine dinucleotide(NAD) combination and catalyzation region. Sir2 can cause target protein deacetylation through NAD dependent manner and it plays an important role in DNA repair, stress resistance and anti-senescence[3]. Silent information regulator 1(Sirt1) is the closest homologous subtype of Sir2 which can cause P53, forkhead transcription factor(FOXO), peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and nuclear factor κ B(NF- κ B) *et al.* deacetylation[4] to reduce ROS production and protect mitochondrial function. The anti-inflammation, anti-OS and anti-apoptosis property of Sirt1 can protect cells from stress damage effectively.

OS is closely related with senescence. The ROS produced during OS can induce the activation of P53, increase the expression of Bax and P21 downstream to cause P53 dependent apoptosis and senescence[5]. P53 is the downstream target of Sirt1. Sirt1 can exert its anti-aging effects by P53 deacetylation and reducing the effects of P53 on its downstream pathways[6]. As a result, Sirt1 is correlated with various aging-related diseases including DM, Alzheimer's disease, cancer, chronic obstruction pulmonary disease, osteoarthritis and osteoporosis(OP). In senescence OP and ovariectomy rats models, the expression of Sirt1 decreased, senescence pathways P53/P21 and P16 were activated, which were correlated with bone metabolic disorder and bone senescence. These results indicated that

Sirt1 could also exert anti-aging function in bone[7,8]. But there are no reports about Sirt1 and senescence pathways in DOP till now.

DM can aggravate senescence OP and even can increase the expression of caveolin-1 which represent senescence gene[9]. Meanwhile, studies also found that the expression of anti-aging gene Klotho decreased in DM patients and rats[10,11]. Klotho is a anti-aging gene discovered by Kuro-o M from Japan in 1997[12]. A defect in Klotho gene expression in mice leads to a premature senility syndrome including osteoporosis. There are two forms of Klotho including the membrane-bound Klotho(mKlotho) in cytoplasm, endoplasmic reticulum and Golgi apparatus inside cells and the secreted Klotho(sKlotho) in body fluids. mKlotho mainly express in renal distal convoluted tubules, brain choroid plexus and parathyroid gland. Fibroblast growth factor-23(FGF-23) can maintain the mineral metabolic homeostasis by regulating calcium, phosphorus, vitamin D and parathyroid hormone(PTH) metabolisms to regulate bone metabolisms indirectly. While mKlotho can act as the cofactor of FGF23 to increase the affinity between FGF23 and FGF receptors(FGFRs) and to enhance the effects of FGF23. sKlotho can act as a kind of cytokine to exert multiple effects including anti-OS and anti-inflammation effects by regulating various cell signaling pathways. Compared with mKlotho, sKlotho is a potential research target recently.

Although there are no report about the relationship between Klotho and DOP, many studies have confirmed the relationship between Klotho and bone metabolism. Klotho^{-/-} mice characterized by abnormal bone metabolism[13]. Both of the osteoblasts and osteoclasts from Klotho^{-/-} mice manifested low differentiated function and the decrease of bone formation was more than the decrease of bone absorption which is the characteristic of senescence OP. The serum of sKlotho was also decreased in senescence OP patients[14]. However, there are no reports about the effects and mechanisms of sKlotho regulating bone metabolism till now.

Previous studies have confirmed that sKlotho could reduce ROS and inflammatory factors by regulating multiple cell signaling pathways such as inhibiting senescence pathway P53/P21[15] in cardiac vascular endothelial system[16], respiratory system[17] and nervous system[18]. We wondered whether sKlotho could exert the anti-OS and anti-aging effects in bone and even in DOP as well.

As we know above, OS is the common pathogenesis of both senescence OP and DOP in which a large number of ROS were produced that could reduce the expression and activity of Sirt1. Sirt1 can exert anti-aging effects to improve bone metabolism in senescence OP. Klotho is a kind of anti-aging gene which is decreased in DM. sKlotho protein encoded by Klotho has the anti-OS effects. Therefore, we speculated that sKlotho could exert its anti-OS effects to up-regulate the expression of Sirt1, inhibit the activation of senescence pathway P53/P21 and P16 to improve the osteoblast function.

Results

2.1 High glucose inhibits the proliferation and differentiation and increases the apoptosis of MC3T3-E1.

We cultured MC3T3-E1 with normal glucose(5.6mmol/L) and high glucose *in vitro*. First of all, three concentrations of high glucose including 15, 25 and 30mmol/L were set in order to screen the optimal stimulated effects on the proliferation and differentiation of MC3T3-E1. CCK8 was performed to detect the proliferation of cells for 24h, 48h and 72h. Western Blot was performed to detect the expression of Runt-related transcription factor 2(Runx2) protein for 120h. ALP microplate test was performed to detect the activity of ALP for 120h. The results of CCK8 showed that 15mmol/L high glucose significantly increased the proliferation of MC3T3-E1 compared with normal glucose group after 24 h, 48h and 72h of exposure ($P<0.05$). There was no significant difference among normal glucose group, 25mmol/L and 30mmol/L high glucose group after 24 h of exposure. However, after 48h and 72h exposure, the proliferation of cells in 25mmol/L and 30mmol/L high glucose groups were significantly decreased compared with normal glucose group($P<0.01$), and the proliferation of cells in 30mmol/L high glucose group was significant decreased compared with 25mmol/L high glucose group($P<0.05$)(Figure 1A). The results of Western Blot and ALP microplate test showed that compared with normal glucose group, the expression of Runx2 protein and the activity of ALP significantly increased in 15mmol/L high glucose group but decreased in both 25mmol/L and 30mmol/L high glucose groups. Moreover, the Runx2 protein expression and the activity of ALP decreased along with the increase of high glucose concentration($P<0.01$) (Figure 1B and 1C). These results indicated that 30mmol/L high glucose concentration could exert the maximum effects on the inhibition of both proliferation and differentiation in MC3T3-E1 among these three concentrations. So we chose 30mmol/L high glucose concentration as the optimal stimulation in the following tests.

In order to eliminate the interference of high osmotic effect, MC3T3-E1 cells were cultured in normal glucose(5.6mmol/L glucose), high mannitol(5.6mmol/Lglucose+24.4mmol/L mannitol) and high glucose(30mmol/L glucose) respectively to observe the effects of high glucose on the proliferation, apoptosis and differentiation of MC3T3-E1. CCK8 was performed to detect the proliferation of cells for 24h, 48h and 72h. The fluorescent dye annexin V-FITC/PI apoptosis detection kit was used to detect the apoptosis of cells for 48h. Western Blot was performed to detect the expression of Runx2, OC and ALP proteins for 120h. The results showed that after 48h and 72h exposure, the proliferation of cells in high glucose groups were significantly decreased compared with normal glucose group($P<0.01$), and the proliferation of cells at 72h was significant decreased compared with 48h exposure($P<0.05$)(Figure 1D). After 48h exposure, the apoptosis of cells in high glucose groups were significantly increased compared with normal glucose group($P<0.01$) (Figure 1E). After 120h exposure, the expression of RUNX2, ALP($P<0.01$) and OC($P<0.05$) were significantly decreased compared with normal glucose(Figure1F). There were no significant differences between normal glucose group and high mannitol group(Figure 1D,1E and 1F).

2.2 High glucose decreases the expression of Sirt1 and activates the senescence pathway P53/P21 and P16.

MC3T3-E1 cells were cultured in normal glucose(5.6mmol/L glucose), high mannitol(5.6mmol/Lglucose+24.4mmol/L mannitol) and high glucose(30mmol/L glucose) respectively

to observe the effects of high glucose on the expression of Sirt1 and P53, acetylated P53, P21 and P16 of MC3T3-E1. Western Blot was performed to detect the expression of pathway proteins above for 72h. The results showed that after 72h exposure, the expression of Sirt1 was significantly decreased and the expression of P53, acetylated P53, P21 and P16 were significantly increased compared with normal glucose ($P < 0.01$). There were no significant differences between normal glucose group and high mannitol group (Figure 2).

2.3sKlotho increases the proliferation and differentiation and decreases the apoptosis of MC3T3-E1 under high glucose.

In this part of our study, we will observe the effects of sKlotho on the metabolisms of MC3T3-E1 under normal glucose and high glucose conditions.

In order to screen the optimal concentration of sKlotho, five concentrations of recombinant sKlotho protein were set including 0.01ug/ml, 0.02ug/ml, 0.1ug/ml, 0.2ug/ml and 1ug/ml. We found that 1ug/ml sKlotho had the lethal effect, so MC3T3-E1 were cultured in high glucose (30mmol/L), high glucose plus sKlotho (0.01ug/ml, 0.02ug/ml, 0.1ug/ml and 0.2ug/ml respectively) in the following studies. CCK8 was performed to detect the proliferation of cells for 72h. Western Blot was performed to detect the expression of Runx2 protein for 120h. ALP microplate test was performed to detect the activity of ALP for 120h. The results of CCK8 showed that compared with high glucose group, the proliferation of MC3T3-E1 significantly increased with the increase of the sKlotho concentration ($P < 0.01$) (Figure 3A). The results of Western Blot showed that compared with high glucose group, the expression of Runx2 protein significantly increased with the increase of the sKlotho concentration and Runx2 expressed the most under high glucose plus 0.2ug/ml sKlotho ($P < 0.01$) (Figure 3B). The results of ALP microplate test showed that compared with high glucose group, the activity of ALP increased the most under high glucose plus 0.1 ug/ml and 0.2ug/ml sKlotho ($P < 0.01$) (Figure 3C). These results indicated that sKlotho could improve the proliferation and differentiation of MC3T3-E1 under high glucose and 0.2ug/ml sKlotho concentration could exert the maximum effects. So we chose 0.2ug/ml sKlotho concentration as the optimal stimulation in the following tests.

Then MC3T3-E1 cells were cultured in normal glucose (NG, 5.6mmol/L glucose), high glucose (HG, 30mmol/L glucose), normal glucose plus 0.2ug/ml sKlotho (NG+KL) and high glucose plus 0.2ug/ml sKlotho (HG+KL) respectively to observe the effects of sKlotho on the proliferation, apoptosis and differentiation of MC3T3-E1 with or without high glucose condition. CCK8 was performed to detect the proliferation of cells for 72h. The fluorescent dye annexin V-FITC/PI apoptosis detection kit was used to detect the apoptosis of cells for 48h. Western Blot was performed to detect the expression of Runx2, OC and ALP proteins for 120h. The results showed that after 72h exposure, the proliferation of cells in HG+KL group was significantly increased compared with HG group ($P < 0.01$) (Figure 3D). After 48h exposure, the apoptosis of cells in HG+KL group was significantly decreased compared with HG group ($P < 0.01$) (Figure 3E). After 120h exposure, the expression of RUNX2, ALP and OC were significantly

increased compared with HG group ($P < 0.01$) (Figure 3F). There were no significant differences between NG group and NG+KL group (Figure 3D, 3E and 3F). These results indicated that sKlotho could improve the metabolisms of MC3T3-E1 under high glucose.

2.4 sKlotho inhibits the senescence pathway P53/P21 and P16.

MC3T3-E1 cells were cultured in normal glucose (NG, 5.6 mmol/L glucose), high glucose (HG, 30 mmol/L glucose), normal glucose plus 0.2 μ g/ml sKlotho (NG+KL) and high glucose plus 0.2 μ g/ml sKlotho (HG+KL) respectively to observe the effects of sKlotho on the expression of P53, acetylated P53, P21 and P16 of MC3T3-E1 with or without high glucose. Western Blot was performed to detect the expression of pathway proteins above for 72h. The results showed that after 72h exposure, the expression of P53, acetylated P53, P21 and P16 in HG+KL group were significantly decreased compared with HG group ($P < 0.01$). There were no significant differences between NG group and NG+KL group (Figure 4). The results indicated that sKlotho could inhibit the activation of senescence pathway P53/P21 and P16 of MC3T3-E1 under high glucose.

2.5 Excessive ROS produced under high glucose decrease the expression of Sirt1

In order to observe the production of ROS under high glucose and the effects of ROS on the expression of Sirt1, we used antioxidant N-acetylcysteine (NAC) to neutralize the excessive ROS produced by high glucose. We cultured MC3T3-E1 in normal glucose (NG, 5.6 mmol/L glucose), high glucose (HG, 30 mmol/L glucose), normal glucose plus 10 mmol/L NAC (NG+NAC) and high glucose plus 10 mmol/L NAC (HG+NAC) respectively. The fluorescent probe DCFH-DA was used to detect the ROS levels for 48h. Western Blot was performed to detect the expression of Sirt1 protein for 72h. The results showed that compared with NG group, the ROS positive cell rate increased and the expression of Sirt1 decreased significantly in HG group ($P < 0.01$) (Figure 5A, 5B). When the ROS was eliminated by NAC in HG+NAC group, the expression of Sirt1 increased significantly compared with HG group ($P < 0.01$) (Figure 5B). These results indicated that excessive ROS produced under high glucose could decrease the expression of Sirt1.

2.6 The effects sKlotho on the expression of ROS and Sirt1 in MC3T3-E1 under high glucose

In order to observe the anti-oxidative effects and the effects on Sirt1 expression of sKlotho with or without high glucose, we cultured MC3T3-E1 in normal glucose (NG, 5.6 mmol/L glucose), high glucose (HG, 30 mmol/L glucose), normal glucose plus 0.2 μ g/ml (NG+KL) and high glucose plus 0.2 μ g/ml sKlotho (HG+KL) respectively. The fluorescent probe DCFH-DA was used to detect the ROS levels for 48h. Western Blot was performed to detect the expression of Sirt1 protein for 72h. The results showed that compared with HG group, the ROS positive cell rate decreased and the expression of Sirt1 increased significantly in HG+KL group ($P < 0.01$). There were no difference between NG and NG+KL group (Figure 6A, 6B). These results indicated that sKlotho could exert its anti-oxidative effect and improve the expression of Sirt1 in MC3T3-E1 under high glucose.

2.7 The improvement of MC3T3-E1 metabolisms and the anti-aging effect of sKlotho under high glucose is regulated by Sirt1

In this part, we will silence Sirt1 gene with small interfering RNA targeting Sirt1 (siRNA-S) to observe the effects of Sirt1 on the metabolism of MC3T3-E1 and senescence pathway P53/P21 and P16 under high glucose and sKlotho.

First of all, in order to confirm the siRNA-S could effectively silence the Sirt1 gene, MC3T3-E1 were transfected with siRNA-S (NG-siRNA-S) and nonspecific control (NG-siRNA-A) respectively. Western Blot were performed to detect the expression of Sirt1 and the results showed that Sirt1 could be inhibited significantly by siRNA-S (Figure 7A).

Then MC3T3-E1 were cultured in normal glucose (NG, 5.6 mmol/L glucose), normal glucose with siRNA-S transfected (NG+siRNA-S, 5.6 mmol/L glucose+siRNA-S), high glucose (HG, 30 mmol/L glucose), high glucose plus 0.2 µg/ml sKlotho (HG+KL) and high glucose plus 0.2 µg/ml sKlotho with siRNA-S transfected (HG+KL+siRNA-S) respectively to observe the proliferation, apoptosis and differentiation of MC3T3-E1 and the expression of senescence pathway proteins including P53, acetyl-P53, P21 and P16. CCK8 was performed to detect the proliferation of cells for 72h. The fluorescent dye annexin V-FITC/PI apoptosis detection kit was used to detect the apoptosis of cells for 48h. Western Blot was performed to detect the expression of Runx2, OC and ALP proteins for 120h. Western Blot was performed to detect the expression of P53, acetyl-P53, P21 and P16 proteins for 72h. The results showed that after 72h exposure, the proliferation of cells in NG+siRNA-S and HG groups were significantly decreased compared with NG group ($P < 0.01$) (Figure 7B). After 48h exposure, the apoptosis of cells in NG+siRNA-S and HG groups were significantly increased compared with NG group ($P < 0.01$), the apoptosis of cells in HG+KL group were decreased compared with HG group while increased in HG+KL+ siRNA-S group significantly compared with HG+KL group ($P < 0.01$) (Figure 7C). After 120h exposure, the expression of RUNX2, ALP and OC in NG+siRNA-S and HG groups were significantly decreased compared with NG group ($P < 0.01$), these differentiated proteins in HG+KL group were increased compared with HG group while decreased in HG+KL+ siRNA-S group significantly compared with HG+KL group ($P < 0.01$) (Figure 7D). After 72h exposure, the expression of ace-P53, P21 and P16 in NG+siRNA-S group were significantly increased compared with NG group but there were no significant difference of P53 expression between these two groups. The expression of P53, acetyl-P53, P21 and P16 in HG group were significantly increased compared with NG group ($P < 0.01$). These signaling pathway proteins decreased in HG+KL group significantly compared with HG group while increased in HG+KL+ siRNA-S group compared with HG+KL group ($P < 0.01$) (Figure 7E). These results showed that the improvement of MC3T3-E1 metabolisms and the anti-aging effect of sKlotho under high glucose could be reversed after silencing Sirt1 gene. That indicated that Sirt1 was a key regulator participating in the osteoblasts metabolisms improvement and anti-aging effects by sKlotho under high glucose.

Discussion

In this study, we confirmed that high glucose could cause the metabolic disorder of MC3T3-E1. Eliminating the influence of high osmotic pressure, high glucose could inhibit the proliferation of MC3T3-E1, increase the apoptosis of MC3T3-E1 and decrease the expression of Runx2, ALP and OC. The results indicate that high glucose can cause the metabolic disorder of MC3T3-E1.

OS is an important pathogenesis of DOP[1]. We found that the ROS increased and the expression of Sirt1 decreased significantly in MC3T3-E1 after 48h cultured in high glucose. We also found that the expression of Sirt1 increased after ROS elimination by antioxidant NAC which indicated that the expression of Sirt1 could be inhibited by excessive ROS under high glucose in MC3T3-E1. The anti-aging mechanism of Sirt1 is closely correlated with its anti-OS effect because large number of ROS produced in OS can induce cellular DNA, lipid and protein damage, telomere shortening and then cause cell senescence[19]. The results of studies about the effects of ROS on the expression and activity of Sirt1 are contradictory. Some studies[20,21] showed that ROS could inhibit the expression and activity of Sirt1 and reduce its deacetylation capacity by reducing NAD⁺ or increasing miR-34a expression. This is consistent with our results that ROS produced under high glucose inhibits the expression of Sirt1. Concerned about the effect of high glucose on Sirt1 expression in the osteoblasts, there is only one study by Gong K[22] reported that the expression of Sirt1 decreased in MC3T3-E1 under high glucose which was consistent with our results.

OS is an important mechanism of cellular senescence. OS can induce senescence by activating multiple signaling pathways including NF- κ B and P38 MAPKs and these pathways can cause senescence pathway P53/P21 and P16 activating finally which indicate the key role of P53/P21 and P16 pathway in regulating cellular senescence[23]. The cell cycle stagnate is the premise of cellular senescence. P21 is the downstream target of P53 and it is widely believed that the P53 dependent cell cycle arrest is mediated by P21 initially. P16 can cause cell senescence because it can influence the phosphorylation of retinoblastoma protein(Rb) and promote cell growth arrest by inhibiting CDK4 and CDK6[24]. The damage of DNA promotes the deacetylation of P53 directly and the deacetylation form of P53 can reduce the degradation of P53, promote the transcriptional activity of P53, increase the production of ROS and accelerate the cellular apoptosis and senescence[25].

Sirt1 can reduce the activity of P53 by deacetylation and inhibit cell apoptosis and senescence associated with P53[3]. Sirt1 can also reduce the expression of P16. Studies have shown the anti-OS, anti-apoptosis and anti-aging effects of Sirt1 in bone metabolism. It has been reported that the expression of Sirt1 decreased and the acetylated P53, Bax and caspase9 increased significantly in MC3T3-E1 stimulated by H₂O₂, and this phenomenon could be reversed by resveratrol which is the activator of Sirt1[26]. Studies showed[8] that the expression of Sirt1 decreased and the genes of P53/P21 and P16 over-expressed in BMSCs from senile osteoporosis rat, which caused the anti-OS ability weakened in BMSCs, and over-expression of Sirt1 could reverse the senescence of BMSCs.

However, there are no report about the role and the interaction of Sirt1 and senescence pathway P53/P21 and P16 in DOP. In our study, we found that the expression of acetylated P53, P21 and P16 increased

accompanied by disorder of MC3T3-E1 (cell proliferation and differentiation decreased, cell apoptosis increased) after Sirt1 gene silenced by siRNA-Sirt1. The results indicated that Sirt1 could regulate the senescence pathway P53/P21 and P16 by deacetylated P53 to improve osteoblast metabolism. Moreover, we found for the first time that the expression of Sirt1 decreased while the expression of P53, acetylated P53, P21 and P16 increased in MC3T3-E1 cultured in high glucose *in vitro*. Studies have found [27,28] that high glucose could cause cellular senescence in renal cells, but the same effects have not been reported in osteoblasts. The results of our study confirmed that excessive ROS produced under high glucose could cause metabolic disorder and senescence of osteoblast by reducing Sirt1 expression *in vitro*.

Klotho is an anti-aging gene encoding two kinds of protein including mKlotho and sKlotho which are distributed in specific tissues and humor respectively. Klotho gene knockout mice manifested bone metabolic disorder which is similar with senile osteoporosis [13].

Previous studies have confirmed that the serum levels of sKlotho decreased significantly in DM and diabetic nephropathy patients [29]. However, there is no report about the relationship between sKlotho and bone metabolism or DOP.

In our study, we found for the first time that sKlotho could improve the function of MC3T3-E1 cultured under high glucose *in vitro*. We found that sKlotho recombinant protein could reduce cell apoptosis and increase the proliferation and the expression of differentiated proteins including Runx2, ALP and OC in MC3T3-E1 under high glucose. Previous studies have confirmed that sKlotho could reduce ROS and inflammatory cytokines by activating FOXOs and cAMP, inhibiting P53/P21 and improving endoplasmic reticulum stress in vascular endothelial system [16], respiratory system [17] and nervous system [18]. We found that sKlotho could reduce the ROS levels effectively which were increased under high glucose. This results indicated for the first time that sKlotho could also exert its anti-OS effect on MC3T3-E1. We have also found that sKlotho could increase the expression of Sirt1 and inhibit the activation of senescence pathway P53/P21 and P16 in MC3T3-E1 under high glucose. Moreover, the improvement of MC3T3-E1 metabolisms and the reverse effect on anti-aging pathway of sKlotho under high glucose could be reversed after silencing Sirt1 gene, which indicated that Sirt1 was a key regulator participating in the osteoblasts metabolisms improvement and anti-aging effects by sKlotho under high glucose. Interestingly, we found that besides the increase of acetylated P53, the expression of P53 increased in HG+KL+Sirt1-siRNA group. We speculated that the elevation of acetylated P53 might prevent the degradation of P53. Another speculation is that the anti-OS effect of Sirt1 [30] decreased after gene silencing and the expression of P53 increased by excessive ROS under high glucose. Concerned about the relationship between sKlotho and Sirt1, a recent study reported that sKlotho could increase the expression of Sirt1 inhibited by monocrotaline, increase the activity of eNOS and reduce the inflammatory reaction [31]. The expression of Sirt1 decreased in the aortic endothelium and smooth muscle of Klotho^{-/-} mice which indicated that Klotho could reduce Sirt1 expression [32]. But the mechanisms were unclosed in these studies. In our study, we confirmed that excessive ROS could inhibit the expression of Sirt1, then we found that sKlotho could reduce ROS and increase the expression of Sirt1. The relationship among

sKlotho, ROS and Sirt1 can be interpreted by different ways. One interpretation is that sKlotho may increase the expression of Sirt1 by reducing ROS. As sKlotho has been found to increase the expression of Sirt1 in endothelial and smooth muscle cells, another interpretation is that sKlotho may increase the expression of Sirt1 by other unknown mechanisms and then Sirt1 exerts its anti-OS, anti-apoptosis and anti-aging effects on MC3T3-E1. Further studies should be carried out to explore more mechanisms.

Conclusion

We found for the first time that high glucose could damage the activity and function of MC3T3-E1, reduce the expression of Sirt1 by inducing OS and activate senescence pathway P53/21 and P16; sKlotho could increase the MC3T3-E1 proliferation and differentiation, exert anti-apoptosis and anti-aging effects via increasing the expression of Sirt1 by inhibiting OS under high glucose. Our study may provide new treatment target for DOP and new research field of sKlotho.

Methods

5.1 Reagents and Cell culture

Mouse recombinant Klotho protein was bought from Cloud-Clone Corporation (Houston, TX, USA). The antibodies used in this study include: rabbit polyclonal anti-ALP (CLOUD-CLONE CORP,USA), rabbit polyclonal anti-OC (CLOUD-CLONE CORP,USA), rabbit polyclonal anti-Runx2 (Santa Cruz Biotechnology, USA), rabbit polyclonal anti-Sirt1 (Santa Cruz Biotechnology, USA), rabbit polyclonal anti-P53 (Cell Signaling Technology,USA), rabbit polyclonal anti-acetylated P53(Cell Signaling Technology,USA), rabbit polyclonal anti-P21(Bioss Inc, China), rabbit polyclonal anti-P16(Bioss Inc, China), rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, USA).

The mouse fetal osteoblastic cell line MC3T3-E1 was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Science, China. Cells were grown in α -MEM medium (HyClone, USA) supplemented with 10% fetal bovine serum (PAN, Germany) in a humidified 5% CO₂ atmosphere at 37°C. The medium was changed every other day and the cells were sub-cultured with trypsin-EDTA.

5.2 Grouping

After pre-incubation in α -MEM without fetal bovine serum overnight, cells were used for subsequent experiments. The cells were treated with one of the following: normal glucose group(NG,5.6mmol/L glucose); high glucose group(HG,15,25 and 30mmol/L glucose); hypertonic group(HM, 5.6mmol/L glucose+24.4mmol/L mannitol); NG +recombinant Klotho protein group(NG+KL, NG+0.2ug/ml KL); HG+ recombinant Klotho protein group(HG+KL, 30mmol/L glucose+0.01,0.02,0.1 and 0.2ug/ml KL); NG+ N-acetylcysteine group(NG+NAC,NG+10mmol/L N-acetylcysteine); HG+ N-acetylcysteine group(HG+NAC, 30mmol/L glucose+10mmol/L N-acetylcysteine); NG+Sirt1-siRNA group(NG+siRNA-S, NG+40nmol/L

Sirt1-siRNA); HG+ KL+Sirt1-siRNA group(HG+KL+ siRNA-S, 30mmol/L glucose+0.2ug/ml KL+40nmol/L Sirt1-siRNA). MC3T3-E1 in NG group were used as the control group.

5.3 Sirt1 siRNA transfection

MC3T3-E1 plated into six well plates were transiently transfected with 100pmol Sirt1 siRNA(sc-40987) (Santa Cruz, CA) or Control siRNA-A(sc-37007) as a negative control by Lipofectamine 2000(Invitrogen, USA). A total of 5 h after transfection, cells were treated with normal glucose or high glucose+recombinant Klotho protein.

5.4 Assay for cell proliferation

Cell proliferation was measured using CCK8 kit(Dojindo,Japan). Briefly, the MC3T3-E1 cells were seeded onto 96-well plates (8000 cells/well) and cultured in a humidified 5% CO₂ atmosphere at 37°C for 24 h. Then the cells were treated with different stimulations for another 24,48 or 72h. Finally, 10ul CCK8 was added into each well and cultured in a humidified 5% CO₂ atmosphere at 37°C for 3h. The optical densities(OD) were measured at 450 nm spectral wavelength using a microplate reader (Spectra Thermo, Switzerland). The experiments were repeated 3 times under the same experimental conditions.

5.5 Cell Apoptosis Analysis

Apoptosis was analyzed by the fluorescent dye annexin V-FITC/PI apoptosis detection kit according to the manufacturer's protocols (Seven Sea, China). In brief, cells stimulated for 48h were harvested and washed in PBS and re-suspended in 400ul 1×binding buffer to ensure 1×10⁵cells/ml, and incubated with 5ul of annexin V-FITC for 15 min at room temperature in the dark. Then 10ul of PI solution were added into the mixed liquor and incubated in ice for 5 min in the dark, and analyzed by bivariate flow cytometry (BD Biosciences,USA) within 30 min. The experiments were repeated 3 times under the same experimental conditions.

5.6 ALP activity detection

Cells stimulated for 120h were harvested and lysed in RIPA lysis buffer containing PMSF protease inhibitors. We collected the cell lysate for the next detection on 96-well plates according to the following manufacturer's protocols(Jiancheng, China)(Table 1).

Table1 The protocols of ALP activity detection

| | Blank well | Standard well | Detection well |
|--|------------|---------------|----------------|
| double distilled water (ul) | 5 | | |
| 0.1mg/ml Liquid phenol (ul) | | 5 | |
| sample(ul) | | | 5 |
| buffer solution (ul) | 50 | 50 | 50 |
| matrix liquid (ul) | 50 | 50 | 50 |
| fully mixed under 37°C water bath for 15 min | | | |
| color agent(ul) | 150 | 150 | 150 |

The optical densities(OD) were measured at 520 nm spectral wavelength using a microplate reader (Spectra Thermo, Switzerland). ALP activity was normalized to the total protein concentration for each sample using a BCA protein assay (Beyotime, China). The experiments were repeated 3 times under the same experimental conditions.

5.7 ROS detection

ROS was detected by fluorescent probe 2,7-dichlorofluorescein diacetate(DCFH-DA) (Beyotime, China). Briefly, the fluorescent probe was prepared by fetal bovine serum-free α -MEM medium and DCFH-DA to make the final concentration of DCFH-DH reach 10 μ mol/L. The MC3T3-E1 cells were seeded onto 6-well plates and stimulated by high glucose or recombinant Klotho protein for 48h. After removing the medium, 1ml DCFH-DA fluorescent probe was added into each well of the 6-well plate and cultured in a humidified 5% CO₂ atmosphere at 37°C for 20 min. The cells were harvested and washed in PBS for three times then re-suspended for final detection by flow cytometry. The experiments were repeated 3 times under the same experimental conditions.

5.8 Protein extraction and Western Blot analysis

Cells were harvested and lysed in RIPA lysis buffer containing PMSF protease inhibitors. Protein concentrations were measured by a BCA assay (Beyotime, China) using BSA as a standard. Samples were boiled at 100 °C for 10 min in 5 \times sample buffer. Equal amounts of protein (40 μ g per sample) were separated by 10% or 15% SDS-PAGE and then transferred onto a PVDF membrane with 200 mA constant current. After blocking with 5% BSA in Tris buffered saline Tween20 (TBS-T, pH 7.6) for 2 h at room temperature, membranes were probed with primary antibodies overnight at 4°C. Primary antibodies for Sirt1, Runx2, ALP, OC, P53, ace-P53, P21, P16 and GAPDH were used at a dilution ratio of 1:500. After extensive washing, the membranes were incubated with anti-rabbit IgG secondary Antibody (1:8000). The membranes were then reacted with ECL-Plus chemiluminescent detection HRP reagents (Beyotime, China). Immunoreactive bands were visualized using a MicroChemi 4.2 Bio-imaging system (Jerusalem, Israel). The experiments were repeated 3 times under the same experimental conditions.

5.9 Statistical analyses

Data obtained from at least three independent experiments are expressed as mean±SD. The IBM SPSS statistics (V.19.0, IBM Corp., USA, 2010) was used for data analysis. Differences between the groups were analysed by ANOVA, followed by LSD's test for normally distributed values. All P-values reported were two-tailed, and P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant.

Declarations

6.1 Ethics approval and consent to participate

Not applicable.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

6.4 Competing interests

The authors declare that they have no competing interests.

6.5 Funding

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6.6 Authors' contributions

Xiaoyu Ma and Can Wu performed the major experiments, and Xiaoyu Ma was a major contributor in writing the manuscript. Fenqin Chen and Xiujuan Lv analyzed the data. Qiuyue Wang was the adviser. All authors read and approved the final manuscript.

6.7 Acknowledgements

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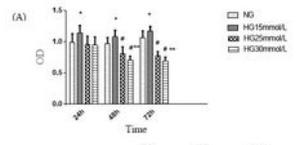
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Figures



(B) NG 15mmol/L 25mmol/L 30mmol/L

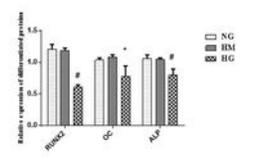
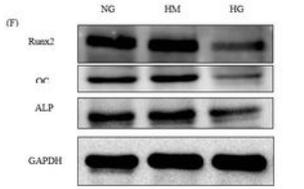
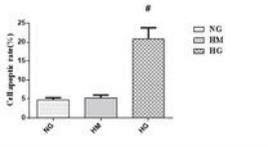
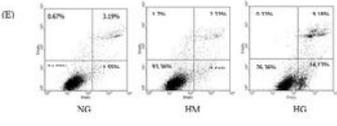
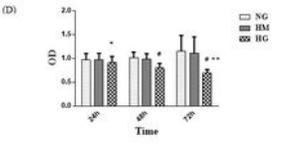
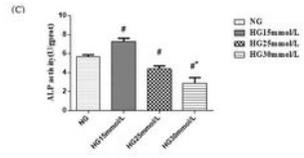
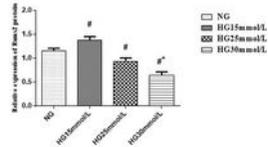
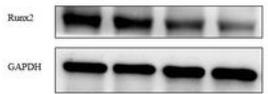


Figure 1

The effects of high glucose on the proliferation, differentiation and apoptosis of MC3T3-E1. (A) The effects of different concentration of high glucose on the proliferation of MC3T3-E1 for 24h,48h and 72h. (B) The effects of different concentration of high glucose on the expression of Runx2 for 120h. (C) The effects of different concentration of high glucose on the activity of ALP for 120h. (D) The effects of high glucose on the proliferation of MC3T3-E1 for 24h,48h and 72h. (E) The effects of high glucose on the

apoptosis of MC3T3-E1 for 48h. (F) The effects of high glucose on the differentiation of MC3T3-E1. The proliferation of cells was detected by CCK8. The apoptosis of cells was detected by the fluorescent dye annexin V-FITC/PI apoptosis detection kit. The expression of differentiated protein including Runx2, OC and ALP proteins were detected by Western Blot. Western Blot results were normalized to GAPDH. The activity of ALP was detected by ALP microplate test. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. (A)Notes: * $P<0.05$, # $P<0.01$ vs NG and ** $P<0.05$ vs HG25mmol/L at each time point (B)Notes: # $P<0.01$ vs NG; * $P<0.01$ vs HG25mmol/L (C)Notes: # $P<0.01$ vs NG; * $P<0.01$ vs HG25mmol/L (D)Notes: # $P<0.01$ vs NG; * $P <0.05$ ** $P <0.01$ vs HG 48h (E)Notes: # $P<0.01$ vs NG (F)Notes: * $P<0.05$, # $P <0.01$ vs NG. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; HM: high mannitol; Runx2:runt related transcription factor 2; ALP:alkaline phosphatase; OC:osteocalcin.

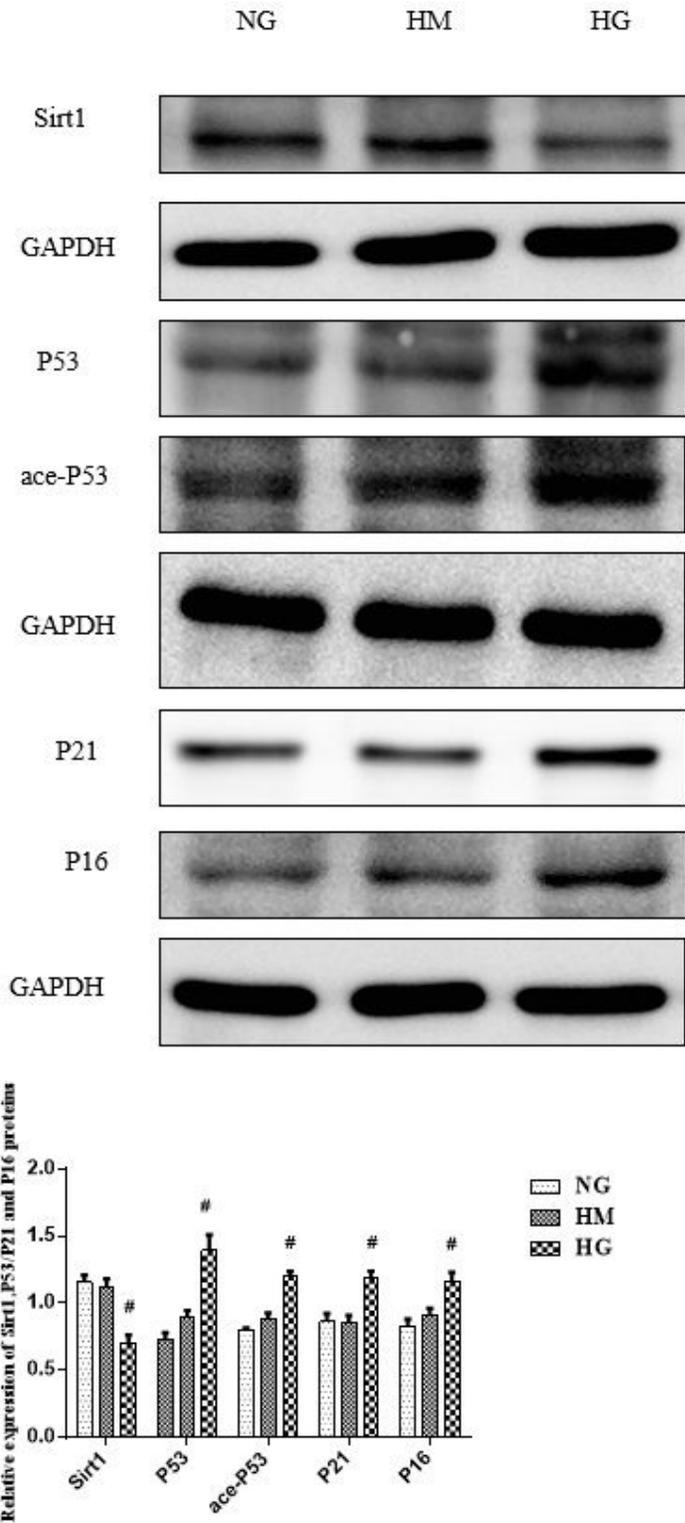


Figure 2

The effects of high glucose on the proliferation, differentiation and apoptosis of MC3T3-E1. (A) The effects of different concentration of high glucose on the proliferation of MC3T3-E1 for 24h,48h and 72h. (B) The effects of different concentration of high glucose on the expression of Runx2 for 120h. (C) The effects of different concentration of high glucose on the activity of ALP for 120h. (D) The effects of high glucose on the proliferation of MC3T3-E1 for 24h,48h and 72h. (E) The effects of high glucose on the

apoptosis of MC3T3-E1 for 48h. (F) The effects of high glucose on the differentiation of MC3T3-E1. The proliferation of cells was detected by CCK8. The apoptosis of cells was detected by the fluorescent dye annexin V-FITC/PI apoptosis detection kit. The expression of differentiated protein including Runx2, OC and ALP proteins were detected by Western Blot. Western Blot results were normalized to GAPDH. The activity of ALP was detected by ALP microplate test. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. (A)Notes: *P <0.05 , #P <0.01 vs NG and **P <0.05 vs HG25mmol/L at each time point (B)Notes: #P <0.01 vs NG; *P <0.01 vs HG25mmol/L (C)Notes: #P <0.01 vs NG; *P <0.01 vs HG25mmol/L (D)Notes: #P <0.01 vs NG; * P <0.05 ** P <0.01 vs HG 48h (E)Notes: #P <0.01 vs NG (F)Notes: *P <0.05 , #P <0.01 vs NG. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; HM: high mannitol; Runx2:runt related transcription factor 2; ALP:alkaline phosphatase; OC:osteocalcin.

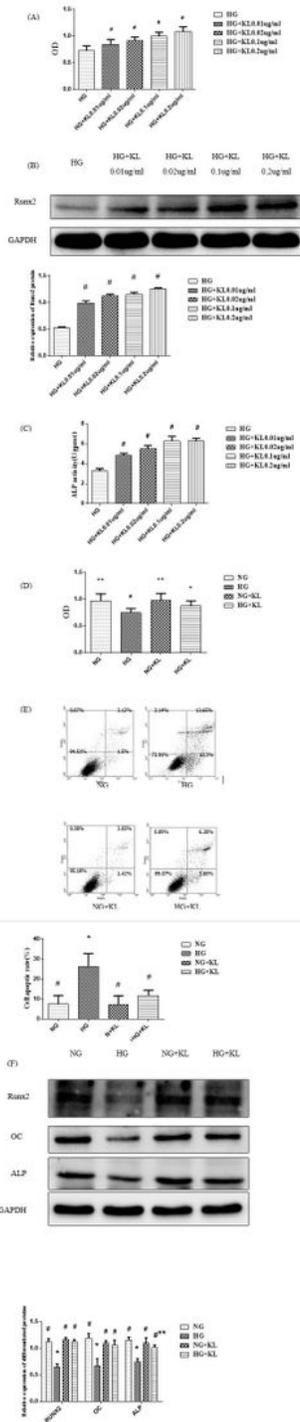


Figure 3

The effects of sKlotho on the proliferation, differentiation and apoptosis of MC3T3-E1. (A) The effects of different concentration of sKlotho on the proliferation of MC3T3-E1 for 72h. (B) The effects of different concentration of sKlotho on the expression of Runx2 for 120h. (C) The effects of different concentration of sKlotho on the activity of ALP for 120h. (D) The effects of sKlotho on the proliferation of MC3T3-E1 for 72h. (E) The effects of sKlotho on the apoptosis of MC3T3-E1 for 48h. (F) The effects of sKlotho on the

differentiation of MC3T3-E1. The proliferation of cells was detected by CCK8. The apoptosis of cells was detected by the fluorescent dye annexin V-FITC/PI apoptosis detection kit. The expression of differentiated protein including Runx2, OC and ALP proteins were detected by Western Blot. Western Blot results were normalized to GAPDH. The activity of ALP was detected by ALP microplate test. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. (A)Notes: #P <0.01 vs HG (B)Notes: #P <0.01 vs HG (C)Notes: #P <0.01 vs HG (D) Notes: #P <0.01 vs NG; *P <0.05 **P <0.01 vs HG (E)Notes: *P <0.01 vs NG; #P <0.01 vs HG (F)Notes:**P <0.05 , *P <0.01 vs NG; #P <0.01 vs HG. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; KL:sKlotho; Runx2:runt related transcription factor 2; ALP:alkaline phosphatase; OC:osteocalcin.

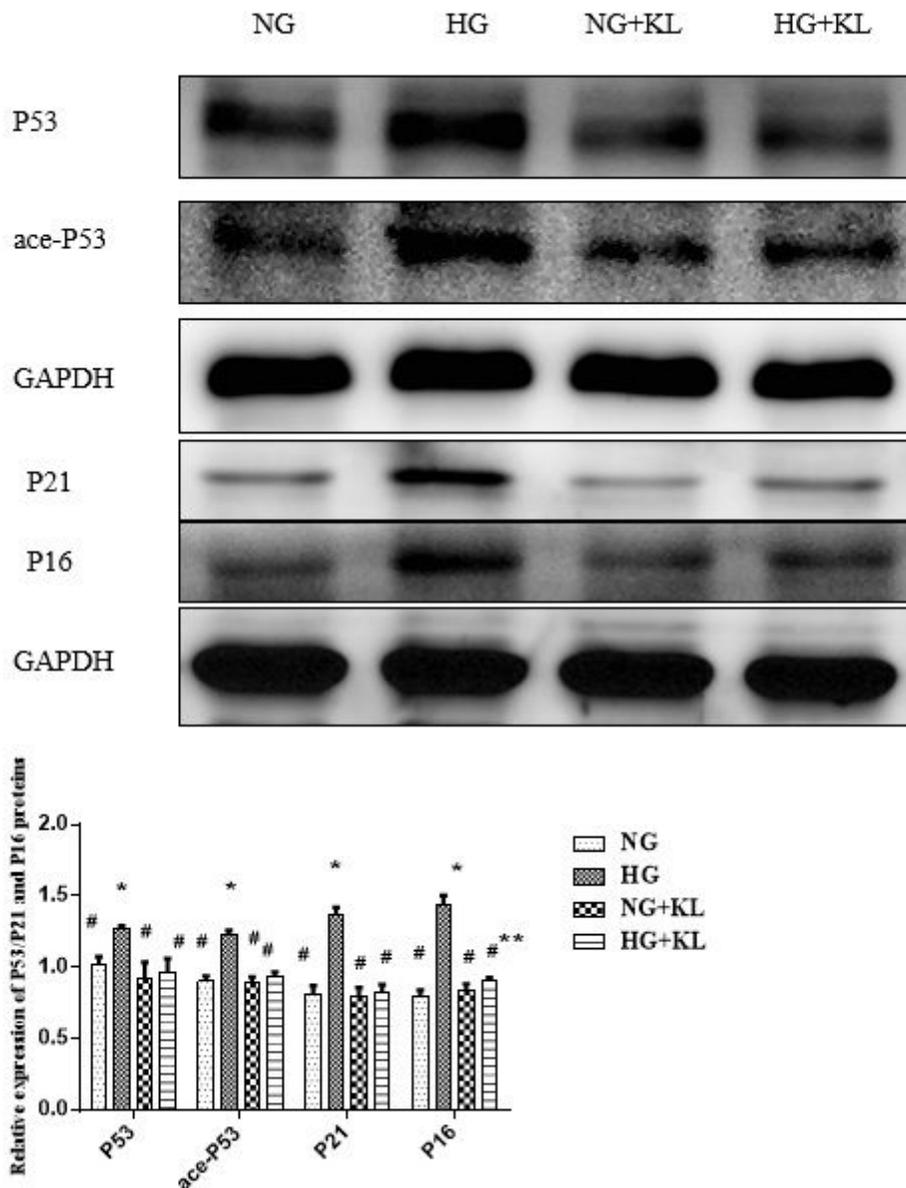


Figure 4

The effects of sKlotho on the senescence signaling pathway P53/P21 and P16 protein expression of MC3T3-E1. The effects of sKlotho on the expression of P53, acetyl-P53, P21 and P16 proteins of MC3T3-E1 for 72h. Notes: **P<0.05, *P<0.01 vs NG; #P<0.01 vs HG. The expression of P53, acetylated P53, P21 and P16 proteins were detected by Western Blot. Western Blot results were normalized to GAPDH. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; KL:sKlotho;ace-P53:acetylated P53.

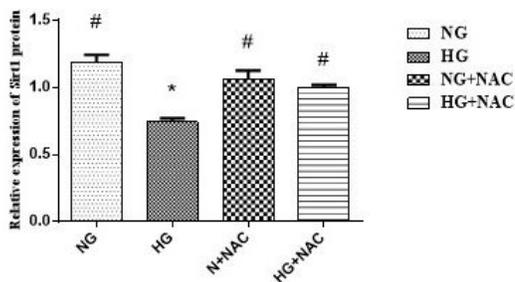
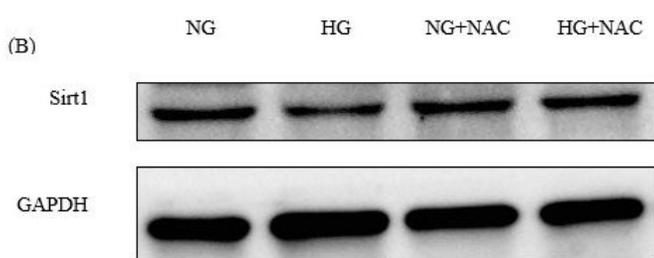
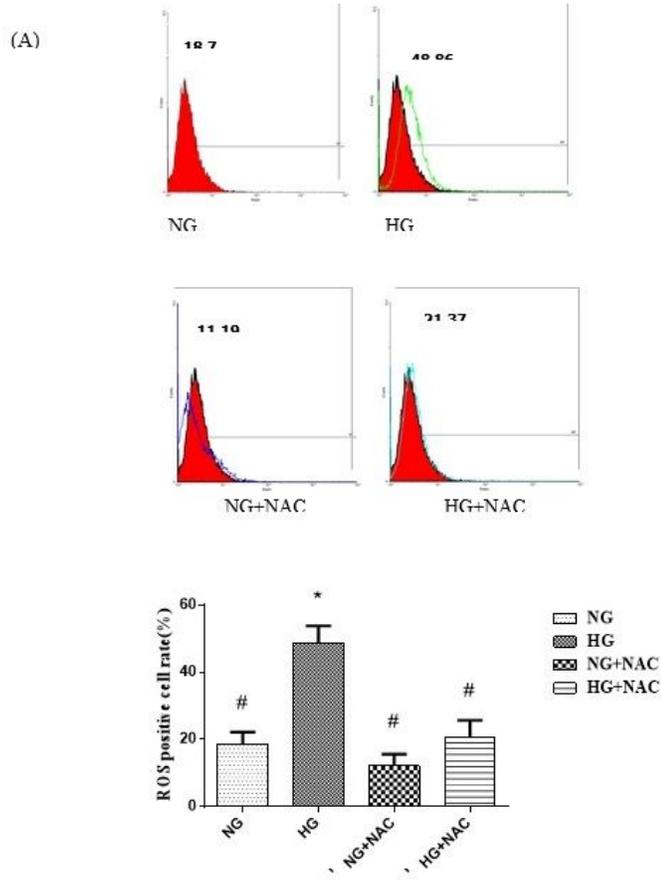


Figure 5

The expression of ROS and Sirt1 in MC3T3-E1 under high glucose. (A)The effects of high glucose on the ROS production for 48h. (B)The effects of high glucose on the expression of Sirt1 for 72h. (A)Notes: *P<0.01 vs NG; #P<0.01 vs HG.(B)Notes: *P<0.01 vs NG; #P<0.01 vs HG. Western Blot results were normalized to GAPDH. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; NAC: N-acetylcysteine; ROS: reactive oxygen species; Sirt1:Silent information regulator 1.

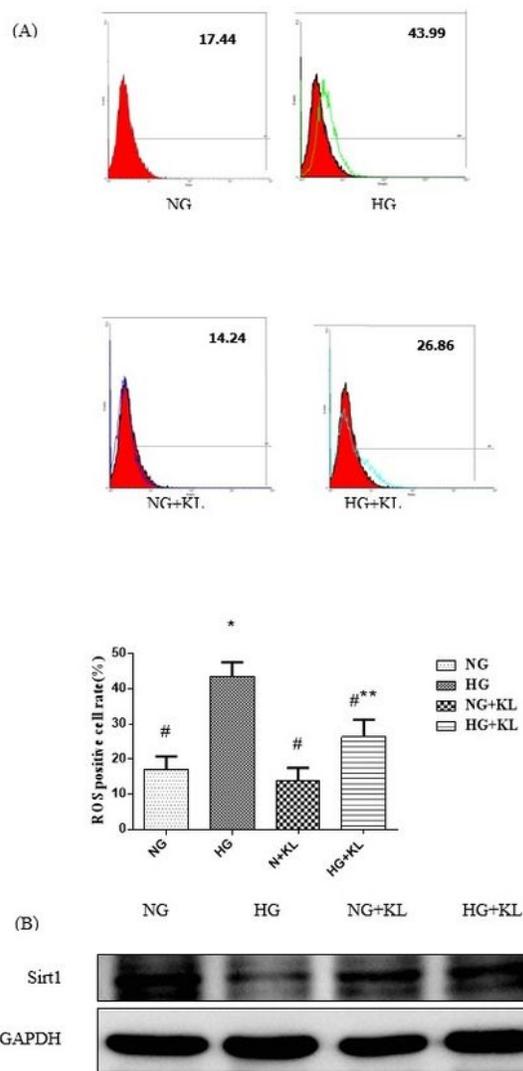


Figure 6

The effects of sKlotho on the expression of ROS and Sirt1 in MC3T3-E1 under high glucose. (A) The effects of sKlotho on the ROS production under high glucose for 48h. (B) The effects of sKlotho on the expression of Sirt1 under high glucose for 72h. (A) Notes: ** $P < 0.05$, * $P < 0.01$ vs NG; # $P < 0.01$ vs HG. (B) Notes: * $P < 0.01$, ** $P < 0.05$ vs NG; # $P < 0.01$ vs HG. Western Blot results were normalized to GAPDH. All results represent means \pm SD obtained from three independent experiments. P-value of < 0.05 was

considered statistically significant, while P-value of <0.01 was highly significant. Abbreviations: NG: normal glucose(5.6mmol/L glucose); HG: high glucose; NAC: N-acetylcysteine; KL: sKlotho; ROS: reactive oxygen species; Sirt1:Silent information regulator 1.

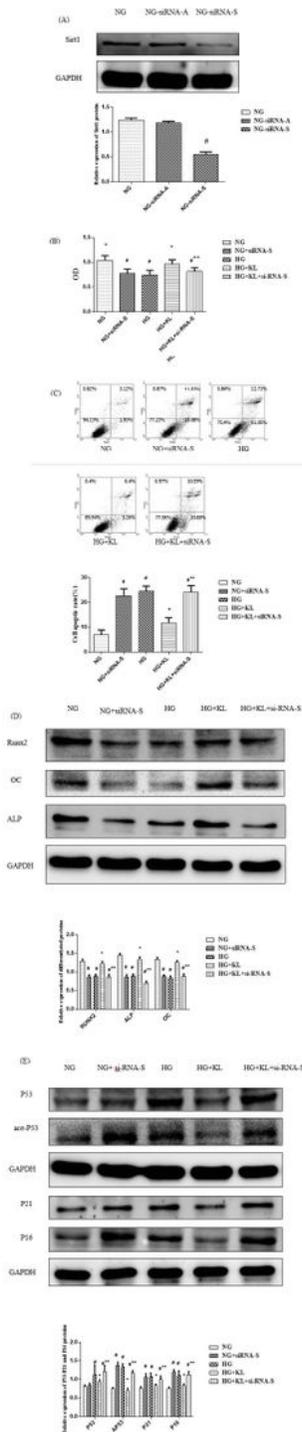


Figure 7

The change of MC3T3-E1 metabolism and senescence pathway P53/P21 and P16 under high glucose and sKlotho after silencing Sirt1 gene. (A)The effects of siRNA-Sirt1 on the expression of Sirt1. (B)The

change of proliferation of MC3T3-E1 under high glucose and sKlotho for 72h after silencing Sirt1 gene. (C) The change of apoptosis of MC3T3-E1 under high glucose and sKlotho for 48h after silencing Sirt1 gene. (D) The expression of Runx2, OC and ALP of MC3T3-E1 under high glucose and sKlotho for 120h after silencing Sirt1 gene. (E) The expression of P53, acetyl-P53, P21 and P16 of MC3T3-E1 under high glucose and sKlotho for 72h after silencing Sirt1 gene. (A)Notes: #P<0.01 vs NG.(B)Notes: #P<0.01 vs NG; *P<0.01 vs HG; **P<0.01 vs HG+KL. (C) #P<0.01 vs NG; *P<0.01 vs HG; **P<0.01 vs HG+KL. (D) #P<0.01 vs NG; *P<0.01 vs HG; **P<0.01 vs HG+KL.(E) #P<0.01 vs NG; *P<0.01 vs HG; **P<0.01 vs HG+KL The proliferation of MC3T3-E1 was detected by CCK8. The apoptosis of cells was detected by the fluorescent dye annexin V-FITC/PI apoptosis detection kit. The expression of Sirt1, Runx2, OC, ALP, P53, acetyl-P53, P21 and P16 proteins were detected by Western Blot. Western Blot results were normalized to GAPDH. All results represent means \pm SD obtained from three independent experiments. P-value of <0.05 was considered statistically significant, while P-value of <0.01 was highly significant. Abbreviations: NG: normal glucose(5.6mmol/L glucose); siRNA-A: nonspecific control; siRNA-S: small interfering RNA targeting Sirt1. HG: high glucose; KL: sKlotho; Sirt1:Silent information regulator 1. Runx2:runt related transcription factor 2; ALP:alkaline phosphatase; OC:osteocalcin.