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The Effects of Uric Acid on Bone Mesenchymal Stem Cells Osteogenic Differentiation

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Abstract: Aims:Investigating the different effects of different concentrations of uric acid on the osteogenic differentiation of human bone mesenchymal stem cells (hBMSCs) and exploring its' mechanism. Methods: Culture in vitro of healthy adult hBMSCs with the method of whole bone marrow adherent. After cultured third generation, respectively cultured hBMSCs in three kinds of medium. MTT colorimetry was used to detected the Proliferation ability of hBMSCs. Observed three groups of hBMSCs morphology with inverted microscope, Compared the control group and the experimental group 1 by RT-PCR, alkaline phosphatase staining(ALP) and alizarin red staining. Immunocytochemistry staining and RT-PCR was used to detected the expression of 11β-HSD1 mRNA , Cbfa1/Runx2 mRNA and Wnt signaling pathway related genes expression. Results: MTT value was significantly higher in the experimental group 1 than in the control group, and has tim- and concentration-dependent; immunocytochemical staining technique showed that in both three groups the cytoplasm cells have brown positive staining granules, detection of 11β-HSD1 mRNA shows that with the concentration of uric acid increased and osteogenic ability increased, optical density decreased gradually, RT-PCR technology showed that both three groups expressed the 11β-HSD1 mRNA, with the concentration of uric acid increased and osteogenic capacity increased, the expression of 11β-HSD1 mRNA gradually decreased; the control group had no Cbfa1/Runx2 mRNA expression in every time point, with the increase of the concentration of uric acid and time prolonged, Cbfa1/Runx2 mRNA expression gradually increased in the experimental group 1; ALP and alizarin red staining showed with the concentration of uric acid increased, the amount of calcium nodules increased; The expression of Wnt signaling pathway related protein Wnt-3a mRNA and β-catenin mRNA was upregulated ($P < 0.05$). Conclusion: uric acid can promote the differentiation of hBMSCs into osteoblasts, which may be accomplished by promoting the expression of 11β-HSD1, Cbfa1/Runx2 and Wnt signaling pathways.

Keywords: Uric acid, Human bone marrow mesenchymal stem cells, Osteogenic differentiation

INTRODUCTION

Human bone mesenchymal stem cells (hBMSCs) is a kind of non hematopoietic stem cells, and is good at self renewal and proliferation in vitro, easy to be isolated and cultured [Sohni, *et. al.*, 2013]. hBMSCs can differentiate into osteoblasts, adipocytes, chondrocytes and endothelial cells under certain conditions, and the process is regulated by many factors [Kim, *et. al.*, 2013]. Providing potential cell source for bone, cartilage and tendon repair in connective tissue and other related diseases, is considered to be a valuable seed cell in bone tissue engineering [Beane, *et. al.*, 2013].

Uric acid is in the form of uric acid salt under physiological PH condition. The level of uric acid in human far higher than other mammals [Keizman, *et. al.*, 2009]. Uric acid has a strong antioxidant effect, so human life expectancy is significantly higher than other kinds of mammals [Nabipour, *et. al.*, 2011].Osteoporosis (OP) seriously affects the quality of life of the elderly, which is a worldwide public health problem [Yaturu, *et. al.*, 2009]. Studies have indicated that the metabolic syndrome (MS) is an independent risk factor for OP, and it is more focused on the study of the effects such as weight [Zhang, *et. al.*, 2009], blood glucose and blood lipid on the bone mass [Luo, *et. al.*, 2008], and there is little research on the relationship between serum uric acid and bone mass. Uric acid is the end product of purine metabolism, a large number of studies have found that uric acid is a risk factor for a variety of diseases, such as cardiovascular disease [Kawai, *et. al.*, 2012], hypertension disease, diabetes [Bandaru, *et. al.*, 2011], metabolic syndrome [Chiou, *et. al.*, 2012] and so on, but the study also found, it is a natural

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antioxidant and free-radical scavenger, scavenging free radicals in the blood, has protective effect on the human body [Spitsin, *et. al.*, 2010]. Recent studies have shown that osteoporosis has a relationship with the oxidative stress, Nabipour et al. have did a survey [Nabipour, *et. al.*, 2010] to 1705 cases over 70 year old elderly in Sydney, Australia, detected their blood uric acid levels and bone mineral density, and found that blood uric acid levels were higher than the average population, their BMD were relatively higher, besides, their vertebral and non-vertebral bone occurred fracture probability reduced greatly.

There are a lot of studies on uric acid, and recent studies have shown that the uric acid that concentration higher than moderate levels can increase bone density and reduce the incidence of osteoporosis, but the specific mechanism is not clear. In our study, we further studied the effects of different concentrations of uric acid on proliferation and osteogenic differentiation of human hBMSCs and its possible mechanism. And we observed the effect that uric acid works on cultured hBMSCs and induced differentiation of bone cells,then observe the change that 11β-HSD1、Cbfα1/Runx2 and Wnt/β-catenin signaling pathway related gene expression have when cultured hBMSCs with uric acid intervented during the differentiation of bone, so as to further explore and discuss the mechanism that uric acid can promote bone formation, and provides theory basis for the clinical use in the bone tissue engineering.

MATERIALS AND METHODS

Main experimental reagents

Low sugar DMEM medium and fetal bovine serum (Gibco); trypsin, uric acid, dexamethasone, vitamin C, alizarin red (Sigma); beta glycerophosphate (Solarbio); alkaline phosphatase Kit (Nanjing Jiancheng Biological Products Limited Company); RNA Extraction Kit (OMEGA). Reverse transcription Kit (FERMENTAS); PCR MIX, 50bp ladder (product code M1041) (Dongsheng Biotechnology Limited Company), Shenggong Biological Engineering (Shanghai) Limited Company design and synthesis primer; immunohistochemistry Rabbit anti 11β-HSD1 antibody (product code ab109554) (ABcam), and the ready-to-use SABC immunohistochemical staining kit (Wuhan boshide company).

Major instruments

Multifunction analyzer, CO2 incubator, super clean bench (Thermo); fluorescence inverted microscope (Nikon); L500 low-speed automatic balancing centrifuge (Changsha Xiangyi); electric thermostatic water bath (Shanghai Jinghong); HZQ-C air bath oscillator (Harbin Dongming); pH meter (Shanghai Jingke); electronic balance (SARTORIUS); multimedia color pathological image analysis system (Shenzhen Swiss Medical Technology Limited Company); DYY-5 electrophoresis (Beijing Liuyi Instrument Factory); Gel-DOC XR gel imager (bio rad company, USA); 50ml cell culture flask, 48 hole cell culture plate, cell culture plates (Corning). Bone marrow from 18-30 years old healthy volunteers, and approved by the ethics committee of our hospital.

Method

Configuration of three kinds of medium: complete medium, uric acid intervented osteogenic culture medium, osteogenic medium.

Isolation and culture the cells: hBMSCs in complete medium as the control group, hBMSCs cultured in uric acid intervented osteogenic culture medium as the experimental group 1, the hBMSCs cultured in the osteogenic medium as the experimental group 2.

MTT colorimetric assay detect BMSCs

Induced hBMSCs differentiation into osteoblasts

Alkaline phosphatase staining and alizarin red staining

Immunocytochemistry staining of osteoblasts

RT-PCR method detect the expression of 11β-HSD1 mRNA, Cbfa1/Runx2 mRNA and Wnt3α/β-catenin mRNA from Wnt signal pathway in different media.

RESULTS

Morphological observation of osteoblasts after induction

In the experimental group 2, the cells were spindle shaped. The cells in the experimental group 1 gradually changed into irregular shape. After that, the convergence of the cells was changed like the paving stones, and the local cells overlapped to form a cell nodule or even a mass. The time of the formation above exist earlier under the uric acid induced than that of the simple osteogenic induction group, and the changes were especially significant with the increase of uric acid concentration and action time. (Figure 1 and 2)

Figure 1 is the morphological observation of cells after 14 days of intervention with different concentrations of uric acid.

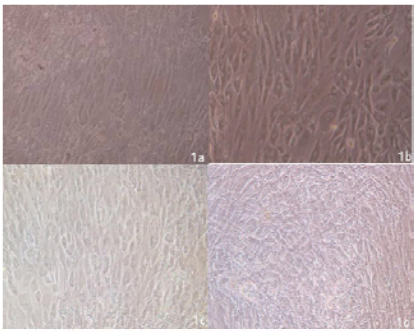

Fig 1: The comparison among passage 3 hBMSCs that induced by defferent medium on the 14th day.(×100)
1a: osteogenic induction medium
1b: 0.2mmol/L uric acid intervention osteogenic induction medium
1c: 0.4mmol/L uric acid intervention osteogenic induction medium
1d: 0.8mmol/L uric acid intervention osteogenic induction medium

Figure 2 is the morphological observation of cells after induced by different days of 0.8mmol/L uric acid intervention.

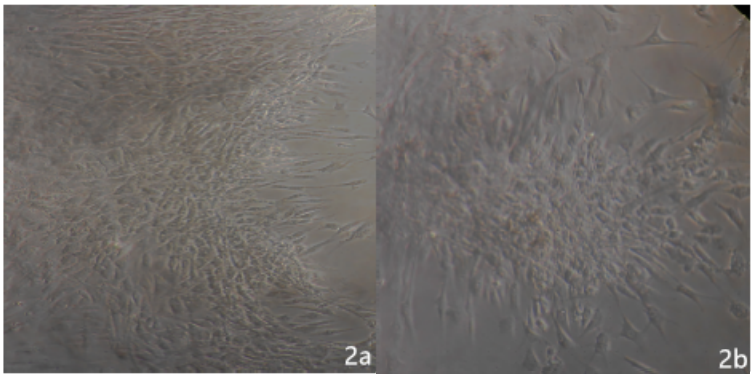

Fig.2: Passage 3 hBMSCs, induced by 0.8mmol/l uric acid intervention osteogenic induction mediums, the comparison between them on the fifth day (fig.2a) and eighth day (fig.2b). (×100)

Effects of different concentrations of uric acid on cell proliferation

The results showed that the OD value of experimental group 1 were higher than control group, and the difference has statistical significance.It means that uric acid can promote hBMSCs proliferation, in a time and concentration dependent manner. According to figure 5, with the increase of concentration hBMSCs the proliferation was increased.

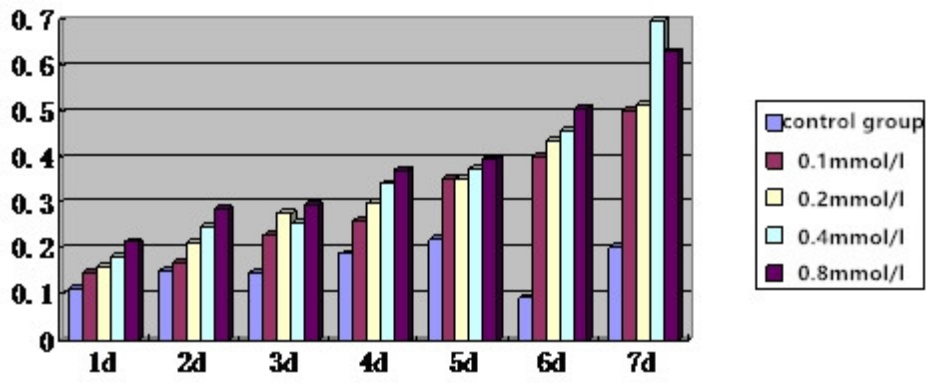

Fig.3 Effect of proliferation of BMSCs under different concentrations of uric acid

ALP staining and alizarin red staining on osteoblasts after induction.

The results showed that after ALP staining, under the optical microscope we can see that cells cultured in osteogenic culture medium stained positive (cytoplasm stained pale purple like), showed that the induced bone cells has endogenous alkaline phosphatase expression. It indicats that the induced cells became bone cells, the control group stained negative; alizarin red staining showed cells cultured in osteogenic culture medium stained positive (indicating the extracellular matrix has the formation of calcium nodules) and the control group stained negative. The amount of calcium nodules in the uric acid intervented osteogenic culture medium was more than that in the osteogenic medium, the highest is in the 0.8mmol/L uric acid intervented osteogenic culture medium ($P<0.05$).

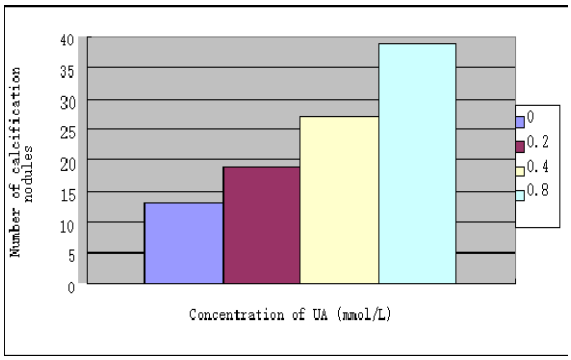

Figure 4：Comparison of the amount of calcium nodules in osteogenic culture medium

11β-HSD1 immunocytochemistry staining results

Microscopically, brown yellow granules were found in the cytoplasm of hBMSCs and osteoblasts of each medium, which indicated the expression of the 11β-HSD1. (Figure 5)

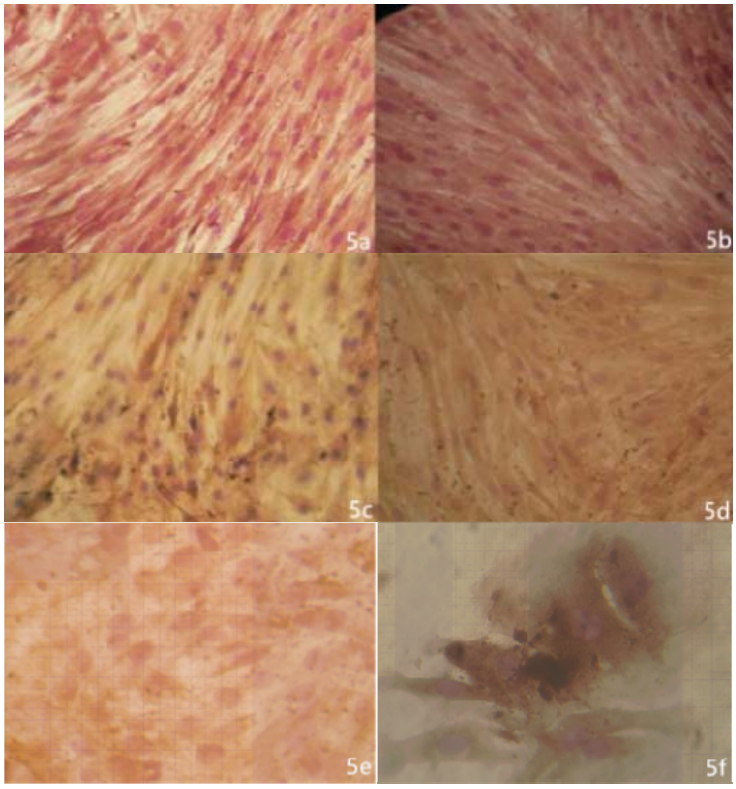

Figure 5：immunocytochmeical result of different mediums of Passage 3 hBMSCs cultured and induced on the 14th day.
5a：complete medium (x100)
5b: osteogenic induction medium(x100)
5c: 0.2mmol/L uric acid intervention osteogenic induction medium(x100)
5d: 0.4mmol/L uric acid intervention osteogenic induction medium(x100)
5e: 0.8mmol/L uric acid intervention osteogenic induction medium(x100)
5f: 0.8mmol/L uric acid intervention osteogenic induction medium(x400)

The expression level of 11β-HSD1

The expression of 11β-HSD1 mRNA in both the experimental group 1 and 2 were lower than the control group,and the expression was descending. ($P<0.05$) (Figure 6).

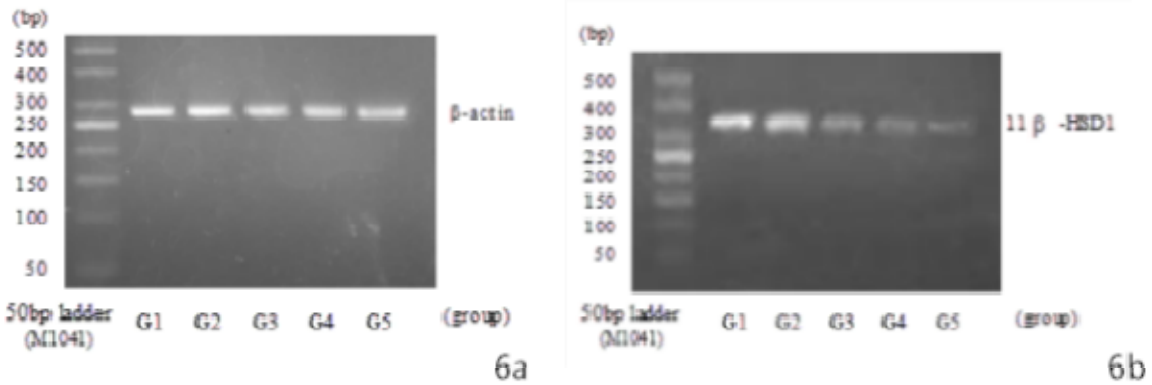

Figure 6：The expression of 11β-HSD1 mRNA in different mediums of Passage 3 hBMSCs cultured and induced on the 14th day.
6a:agarose gel electrophoresis of β-actin (amplified product 285bp) 6b:agarose gel electrophoresis of 11β-HSD1 (amplified product 335bp)
Note: G1（complete medium）
G2（ osteogenic induction medium）
G3（0.2 mmol/L uric acid intervention osteogenic induction medium）
G4（0.4 mmol/L uric acid intervention osteogenic induction medium）
G5（0.8 mmol/L uric acid intervention osteogenic induction medium）

The expression level of Cbfa1/Runx2

RT-PCR detection showed that there is no Cbfa1/Runx2 gene expression in the control group,in the experimental group 1, with uric acid concentration increased and time prolonged, the expression of Cbfa1/Runx2 gene was gradually

increased, and the 0.8mmol/l uric acid concentration is most obviously, and in the same concentration, the gene expression in the 14th day was stronger than that in the 7th day, the differences between groups were statistically significance. ($P < 0.05$) (fig.7)

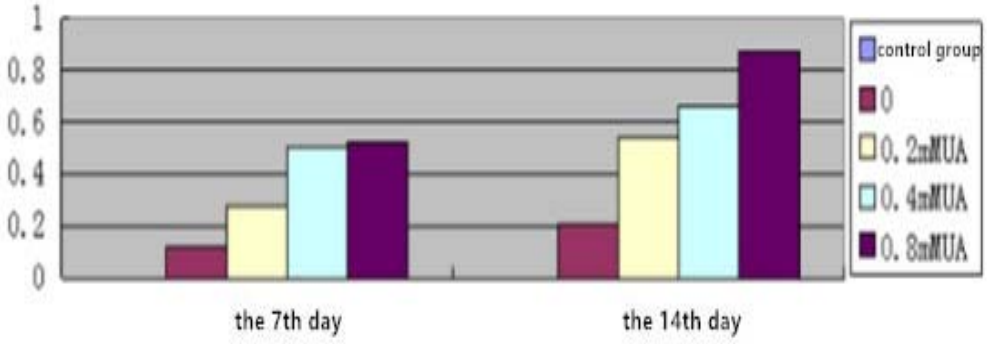

Fig.7 The expression of Cbfa1/Runx2 mRNA in different concenrtate of UA

The expression of Wnt-3a、β-catenin mRNA in different culture medium

The results of RT-PCR showed that the expression of Wnt-3a and β-catenin mRNA in the control group was 1. The expression of Wnt-3a and β-catenin mRNA in the was up-regulated in the experimental groups 1, and the expression of Wnt-3a and β-catenin mRNA increased with the increase of uric acid concentration. Compared with each group, the difference was statistically significant. ($P < 0.05$) (Figure 8)

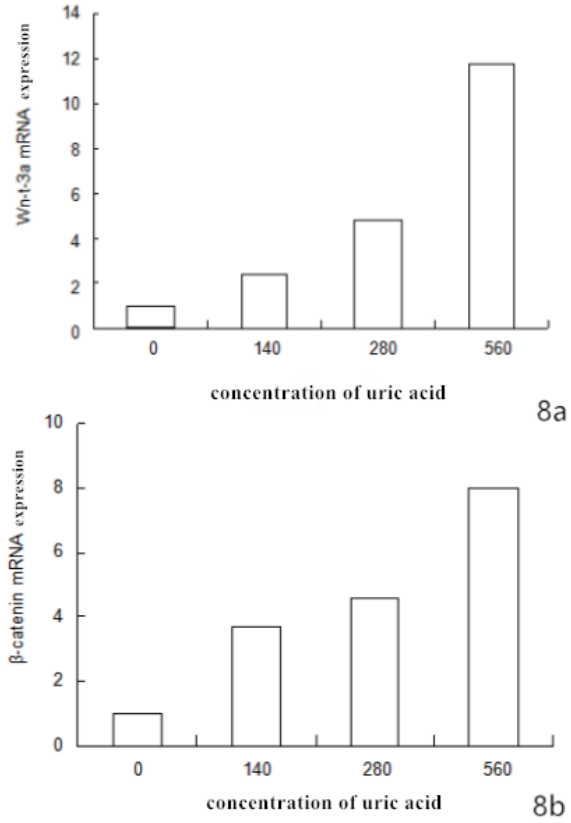

Fig. 8 Effects of different concentrations of uric acid on Wnt-3a mRNA (fig.8a) and β-catenin mRNA (fig.8b) expression during the process of osteogenic differentiation of hBMSCs

DISCUSSION

BMSCs is one of the seed cells in the tissue engineering research. Under normal circumstances, it is in the undifferentiated state of equilibrium, and retains the ability to differentiate into a variety of progenitor cells [8]. Under certain conditions, BMSCs can differentiate into osteoblasts, adipocytes, chondrocytes, nerve cells and so on. Nuttall et al. [9] have found that there is a correlation between the osteogenic and the differentiation of BMSCs, and the occurrence of osteoporosis is related to the decrease of BMSCs osteogenic differentiation ability. Bone marrow mesenchymal stem cells osteogenic differentiation is the most important part of bone formation and bone remodeling, and are subject to the regulation of a variety of local factors and hormone system. These factors and hormones control bone cells in different stages of differentiation [10-11].

The experimental set up different concentration of uric acid, using MTT colorimetric method to determinate their respective OD value.It shows that uric acid can promote the proliferation of BMSCs, and depends on a concentration and time. Therefore, we can conclude that while uric acid promotethe proliferation of BMSCs ,it also can promote the osteogenic induction of BMSCs, and there is a time and concentration dependence. So we can infer that uric acid should be a protective factor of osteoporosis, with increasing levels of uric acid, osteoporosis incidence rate should decline, clinical investigation result of the Nabipour et al. [Nabipour, et. al., 2010] is coincide with this.

The positive staining of ALP was considered to be a specific sign of osteoblast, and it was one of the markers of bone marrow mesenchymal stem cells differentiating into osteoblasts. After induction of 7 and 14 days respectively, ALP and alizarin red staining the cells to identify the osteoblasts. And we can found that ALP activity increased with the concentration of uric acid increasing, and in the

experimental group 1 alkaline phosphatase activity were significantly higher than that of the control group. The observation of cell morphology was in agreement with the biochemical results of alkaline phosphatase activity assay. Therefore, we can conclude that uric acid induced cell is a bone cell, and with the increase of uric acid concentration, the more osteoblast cells are induced. Therefore, we further designed experiments to verify the effect of uric acid on the differentiation of hBMSCs. The experiment is divided into three parts.

In the first part, we studied 11β-HSD1, the activity level of 11β-HSD1 directly affects the glucocorticoids concentration. This experiment conducted the immunocytochemical staining, reverse transcription polymerase chain reaction (RT-PCR) to obtaine the following conclusions: in vitro uric acid, downregulated the expression of 11beta-HSD1, thus can promote hormone induced hBMSCs differentiat into osteoblast, and there is a certain concentration dependence and time dependence.

In the second part, we studied Cbfa1/Runx2. Cbfa1/Runx2 is highly expressed in the osteogenic site, which determines the occurrence and differentiation of osteoblasts. It is a common signaling molecule that induces the osteogenic differentiation [23-26]. The results showed that in the Cbfal gene knockout mice led to the retardation of development of bone tissue, and even the ossification of the cartilage and ossification of the skeleton [27]. This experiment showed that the control group had no Cbfa1/Runx2 mRNA expression, and each experimental group had Cbfa1/Runx2 expression, and the expression of Cbfa1/Runx2 mRNA increased with uric acid concentration increasing and time prolonging, showed concentration and time dependent manner, and the difference between the groups has statistical significance ($P < 0.05$), which we speculate that uric acid may be through the promotion of the expression of Cbfa1/Runx2 gene, so as to promote the osteogenic differentiation of hBMSCs.

In the third part, we studied Wnt-3a/β-catenin signaling pathway. Wnt proteins has unique mode of secretion in the embryonic development and tumor formation they regulate the cell proliferation, differentiation, polarity and a series of functions. Recent studies have indicated that Wnt protein and its downstream signaling pathway play an important role in the proliferation and differentiation of mesenchymal stem cells. In bone cells,Wnt protein is the initiator of the canonical Wnt signaling pathway, can influence the proliferation and differentiation of osteoblasts, in which Wnt-3a molecules play an important role in the differentiation of bone cells, so as to promote BMSCs differentiat into osteoblasts, especially on the expression of alkaline phosphatase regulation. β-catenin is the key factor in the classical Wnt pathway, thus plays an important role in bone formation, a study found that in the β-catenin knockout mice, cortical bone and cancellous bone decreased significantly when mesenchymal precursor cells differentiate into cartilage cells, while promoting the differentiation of osteoclasts and even osteoporosis. results of Wnt-3a、β-catenin mRNA showed that the expression of Wnt-3a and β-catenin enhanced with the increase of uric acid concentration, and has concentration dependence.

In summary, uric acid can promote hBMSCs to differentiate into Osteoblast by promoting the expression of 11beta-HSD1 gene、CBF alpha 1/Runx2 gene and Wnt/β-catenin signaling pathway Wnt-3a and β-catenin gene expression mechanism.

CONCLUSION

Uric acid can promote the differentiation of hBMSCs osteogenic differentiation, which may be accomplished by promoting the expression of 11β-HSD1, Cbfa1/Runx2, and Wnt signaling pathways.

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