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ORIGINAL CONTRIBUTION

A role for suppressed bone formation favoring catch-up fat in the pathophysiology of catch-up growth after food restriction

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Abstract
Purpose Catch-up growth is always companied with later development of obesity and osteoporosis that are two interrelated clinical entities. However, the potential mechanism of the link between them during catch-up growth is unknown.
Methods Rats were divided into two groups. Rats of the normal control (NC) group were offered ad libitum access to food, while rats of CUGFR group were food restricted for 4 weeks, and then were allowed full access to food for 0, 2, 4 weeks, respectively. The fat percentage and distribution, bone mineral density, biochemical and histological indexes of bone were detected. Moreover, the expression of adipogenic or osteoblastic differentiation-related genes of mesenchymal stem cells (MSCs) was also determined.
Results Catch-up growth led to a rapid visceral fat accumulation. Although there was no difference in the histological indexes of bone between NC group and CUGFR group, the bone turnover marker, serum Bone Gla-protein (s-BGP), decreased in CUGFR group. The adipogenic differentiation-related gene of MSCs, PPAR-gamma, was significantly higher than that of NC group especially when catch-up growth for 4 weeks. Nevertheless, the osteoblastic differentiation-related gene of MSCs, Runx2, was increased but failed to reach the levels of the controls eventually. Both protein and mRNA of TAZ, a main transcriptional modulator of MSCs differentiation, failed to catch up even after being allowed full access to food for 4 weeks.
Conclusion CUGFR induces the differential differentiation of MSCs, potentially suppressing bone formation and favoring catch-up fat, which might be responsible for the increased risk of osteoporosis and obesity during CUGFR.

Keywords Catch-up growth · Obesity · Osteoporosis · Mesenchymal stem cells

Background

Catch-up growth, a process that embodies an accelerated recovery from the detrimental effects of poor growth, is increasingly viewed as a long-term health hazard [1], with high susceptibility for central obesity, osteoporosis, type 2 diabetes, and cardiovascular diseases [2, 3]. However, the nature of the link between catch-up growth and risks for such chronic diseases remains obscure.

It was previously believed that obesity and osteoporosis, two widespread diseases with major social and economic impacts, were two independent clinical entities, but recent studies have shown that both diseases may in fact be pathophysiologically related and share several common genetic and environmental factors [4]. Further studies have shown that adipocytes and osteoblasts derive from a common progenitor—the mesenchymal stem cells (MSCs) [5].

How MSCs exit from the cell cycle and differentiate into alternative cell fates such as osteoblasts and adipocytes is incompletely understood. It had been recently discovered that a WW domain-containing molecule, TAZ, functions as a transcriptional modulator to stimulate bone development while simultaneously blocking the differentiation of MSCs

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into fat cells. These developmental effects occur through direct interaction between TAZ and the transcription factors Runx2 or $PPAR_{gamma}$, resulting in transcriptional repression or enhancement, representing selective programs of gene expression [6].

Taken together, these studies above underscore the possible mechanisms of the relation between obesity and osteoporosis. The individuals who have undergone catch-up growth are at a higher risk of developing obesity and osteoporosis compared with those who did not experience catch-up growth, although the exact underlying mechanisms are not fully understood. Whether catch-up growth changes the MSCs differentiation and consequently, alteration in the number of adipocyte and osteoblast though modulating TAZ remains unclear. Therefore, the aim of this study was to examine the impact of catch-up growth after food restriction (CUGFR) on the MSCs differentiation and to indicate a role for suppressed bone formation favoring catch-up fat in the pathophysiology of catch-up growth after food restriction.

Methods

General study design

Six-week-old male Sprague–Dawley rats (Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology, China), weighing 180–200 g and caged singly in wire-bottomed cages in a temperature-controlled room (22 ± 1 °C) with a 12-h light/dark cycle, were raised on a commercial pellet diet (Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology, China) consisting, by energy, of 22% protein, 66% carbohydrates, and 12% fat and had free access to tap water. Animals were maintained in accordance with regulations of the institute and guidelines for the care and use of laboratory animals. The experiments were conducted after 1-week adaptation to housing conditions. All protocols of animal treatment were approved by the institutional animal ethics committee.

Animal model of catch-up growth

Seven-week-old rats ($n = 48$) were randomly divided into two groups: a normal chow group (NC group; $n = 24$) and a CUGFR group ($n = 24$). Rats of NC group were raised on a pellet diet ad libitum for 8 weeks, while rats of CUGFR group were put on food restriction for 4 weeks at 60% of the diet intake of ad libitum-fed rats and then were fed ad libitum for 4 weeks. Changes in food intake were determined once a day, and body weight was measured every 4 days. Eight rats in each group were sacrificed by decapitation 0 (the end of 4-week food restriction), 2 (catch-up growth for 2 weeks), and 4 (catch-up growth for 4 weeks) weeks after re-feeding, and their naso-anal lengths were measured before sacrifice. This animal model has been published previously [7].

Body fat percentage and distribution

At the end of this study, the rats were sacrificed by decapitation. White adipose tissues (subcutaneous, perirenal, epididymal, omental) were carefully dissected out and weighed immediately. The body fat content referred to the real weight of each fat. A rat's total body fat percentage is set to 100%. Body fat percentage of each adipose tissue was determined by dividing the weight of the calculated fat by the total body fat weight.

Measurement of bone mineral density

As described [8], bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DEXA) using a regular XR-36 dual-energy X-ray absorptiometry bone scanner (Norland Co., USA) adapted to measurement in small animals. In briefly, during the measurements, the animals were anesthetized with ether and then the BMD of lumbar spine and tibia were recorded at three different skeletal sites. The lumbar spine was scanned at the levels of the vertebrae L2-4 [9]. The tibia was divided into four equal regions to take into account possible differences in the local proportion of trabecular and cortical bone. BMD was taken as the average of five scans made on each sample.

Detection of biochemical indexes of bone metabolism

The bone turnover markers, serum Bone Gla-protein (s-BGP) and serum Tartrate-resistant Acid Phosphatase (s-TRAP-5b), were detected by ELISA with a rat BGP ELISA kit and a rat TRACP-5b ELISA kit, respectively. Both kits were purchased from Cusabio Biotech Co., Ltd. (Hubei, China), and the minimum detectable dose was typically less than 6 pg/ml for the former and less than 0.02 mIU/ml for the latter. The sensitivity of these two assays, or lower limit of detection (LLD), was defined as the lowest protein concentration that could be differentiated from zero.

Histological examinations of trabecular bone

Animals received an intraperitoneal injection of pentobarbital (30 mg/kg body weight) for anesthesia and prepared the tibia for histological evaluation. Vertical and cross sections were cut. Sections were stained with hematoxylin

and eosin [10]. Images were recorded on a digital camera (Coolpix 950, Nikon, Japan), and an Image Pro Plus software package (Media Cybernetics, Bethesda, MD, USA) was used to measure trabecular width and trabecular separation. Mean trabecular width (MTW) and mean trabecular separation (MTS) were calculated by the mean of 10 trabecular width or trabecular separation at the vertical-sectional level.

Isolation and enrichment of MSCs

As described [11], MSCs were isolated from rat femurs and tibias. The isolated cells were further enriched against two MSCs surface markers CD54 and CD90 by magnetic cell sorting. Expression of surface markers CD54 and CD90 was then confirmed by flow cytometry analysis. The hematopoietic stem cells (HSCs) marker CD45 was used to confirm that the MSCs were depleted of HSCs. Results indicated that the MSCs show positive staining against MSCs markers CD54 and CD90 but negative against the HSCs marker CD45 (Fig. 1), which verified that the enrichment of MSCs was achieved.

RT-PCR and quantitative real-time PCR

Total RNA was extracted from MSCs by using Simply P total RNA extraction kit (BioFlux, Hangzhou, China). Reverse transcription was performed with 1-μg of total RNA as sample material and oligo$(dT)_{20}$ as a primer by using the first strand cDNA synthesis kit (Toyobo Co., Ltd., Osaka, Japan). The resultant cDNA was amplified using primers (synthesized by Sangon Inc. Shanghai, China), and PCR reaction was performed in a thermal cycle. Expression of the Runx2, $PPAR_{gamma}$, TAZ gene was first detected by RT-PCR, and then, real-time quantitative PCR was performed using the following profile. Primer sequences are provided in Table 1. The two-step real-time quantitative PCR was done by using the SYBR Premix ExTaq (TaKaRa Biotechnology Co. Ltd., Dalian, China) and was heated at 95 °C for a minimum time of 30 s, then 40 cycles at 95 °C for 15 s, and 60 °C for 45 s in a DNA iCycler apparatus (BIO-RAD, Mississauga, ON, Canada). The gene expression levels were normalized by a housekeeping gene, beta-actin, firstly, and then, the results were presented as the relative expression level, which were fold ratio to the NC0 group (set to 1).

Western blot analysis

MSCs were lysed in SDS lysis buffer-containing protease inhibitors. The protein concentrations of extracts were measured using a bicinchoninic acid assay kit (Pierce, Rockford, IL), and 25–75 μg was added to 12% polyacrylamide gels. Gels were transferred to nitrocellulose membranes, blocked with a solution of Tween 20 and low-fat milk, and incubated with primary antibodies directed against the TAZ (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200). Labeled protein was detected using horseradish peroxidase-linked goat antirabbit or antimouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000) as appropriate with an ECL detection kit (GE Healthcare-Biosciences, Piscataway, NJ). For quantification of band intensity, appropriate film exposures were scanned and the density of bands determined with an Image Pro Plus software package (Media Cybernetics, Bethesda, MD, USA).

Statistical analysis

All data are expressed as mean ± SD. Statistical analysis between and within groups was assessed by ANOVA using SPSS Software (SPSS13.0, Inc., Chicago, IL, USA). Significance was established to be at the $P \leq 0.05$ level.

Results

Food intake, body weight, and naso-anal length

The average food intake of CUGFR rats was ~40% lower during food restriction and similar to their age-matched

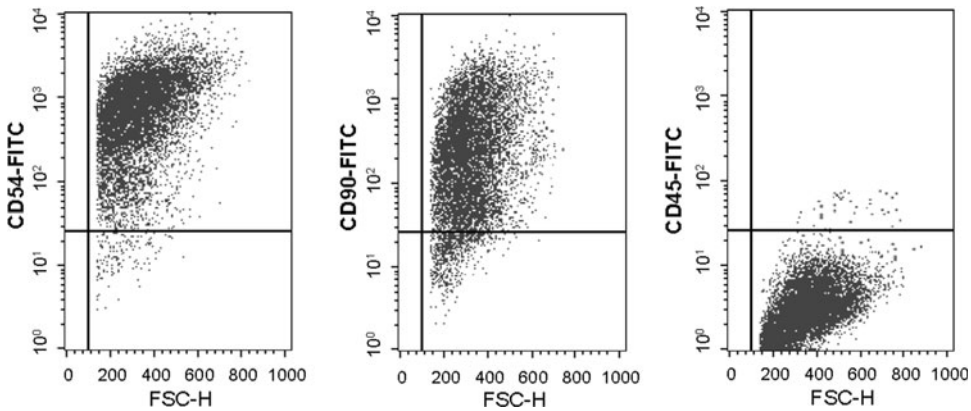

Fig. 1 Flow cytometry analysis of MSCs for surface markers. MSCs were labeled with antibodies against CD45, CD54, and CD90 and detected with flow cytometry

Table 1 Primers used for RT-PCR and quantitative real-time RT-PCR

Gene	Base pairs (bp)	Sequence	Annealing temperature (°C)
Runx-2	150	Sense 5′-CCCAGCCACCTTTACCTACA-3′	58
		Antisense 5′-TATGGAGTGCTGCTGGTCTG-3′	
$PPAR_{gamma}$	525	Sense 5′-ATAAAGTC CTTCCCGCTGACCAAAGCC-3′	60
		Antisense 5′-GCGGTCTCCACTGAGAATAATGAC-AGC-3′	
TAZ	337	Sense 5′-GTCACCAACAGTAGCTCAGATC-3′	60
		Antisense 5′-AGTGATTACAGCCAGGTTAGAAAG-3′	
$Beta_{actin}$	260	Sense 5′-GAGACCTTCAACACCCCAGCC-3′	60
		Antisense 5′-AATGTCACGCACGATTTCCC-3′	

normal chow after re-feeding (Fig. 2a). The body weight and naso-anal length of these rats, however, were consistently lower than that of the control group, even 4 weeks after the re-feeding (Fig. 2b, c).

Body fat content and distribution

Food restriction resulted in a significant decrease in body fat content ($P < 0.05$). Following re-feeding, body fat content, especially the perirenal and omental fat, increased significantly and was higher than the level of NC group when 4 weeks after the catch up ($P < 0.05$). Although subcutaneous fat and epididymal fat also increased to a certain extent, the effect of catch-up growth on them was relatively less. (Fig. 3a).

A remarkable decrease in the body fat percentage of omental adipose tissue was detected during food-restriction phase ($P < 0.05$); however, it increased rapidly following re-feeding. The body fat percentage of epididymal adipose tissue increased during food restriction and decreased gradually with the extent of catch-up growth ($P < 0.05$). However, the body fat percentage of perirenal adipose tissue had a little decrease during food restriction and increase gradually with the extent of catch-up growth ($P < 0.05$). Only body fat percentage of subcutaneous adipose tissue remains almost the same during both food-restriction and re-feeding phase (Fig. 3b).

As shown in Fig. 3a, b, catch-up growth after food restriction resulted in a significant change of body fat content, especially omental and perirenal fat. These results indicated that, from the point of body fat distribution, catch-up growth leads to a rapid visceral fat accumulation.

Bone mineral density

Bone mineral density (BMD) at the level of lumbar spine was lower than that of NC group at the food-restriction session as well as after the catch-up growth for 2 weeks, it reached virtually the same level as that of the control group when catch-up growth for 4 weeks (Fig. 4a).

Although the experimental data over the course of the catch-up growth revealed that there was slightly decrease in the BMD of tibia in comparison with NC group, there was no significant difference between them when catch-up growth for 0, 2, and 4 weeks (Fig. 4b).

Biochemical indexes of bone metabolism

Figure 5a showed that there was lower s-BGP in CUGFR group than in NC group at the end of food restriction. Although catch-up growth partially moderated this trend, the s-BGP in CUGFR group failed to catch up with those of NC group even after the catch-up growth for 4 weeks.

Figure 5b showed that there were all no significant difference of s-TRAP level when compared CUGFR with NC group when catch-up growth for 0, 2, and 4 weeks.

Histological examinations of trabecular bone

Figures 6 and 7 showed that there were all no significant difference in trabecular bone microstructure between NC and CUGFR group, which was observed at the vertical-sectional level and cross-sectional level of rats' femurs, respectively, with hematoxylin and eosin staining.

The adipocyte or osteoblastic differentiation-related gene expression of MSCs

To identify the adipocyte or osteoblastic differentiation of MSCs, some related gene expressions were detected. RT-PCR analysis demonstrated that, Runx2, a key transcription factor associated with osteoblastic differentiation of MSCs, decreased in food-restriction phase and failed to return to the level of control group even after catch-up growth for 4 weeks (Fig. 8a, c1). PPAR-gamma is a

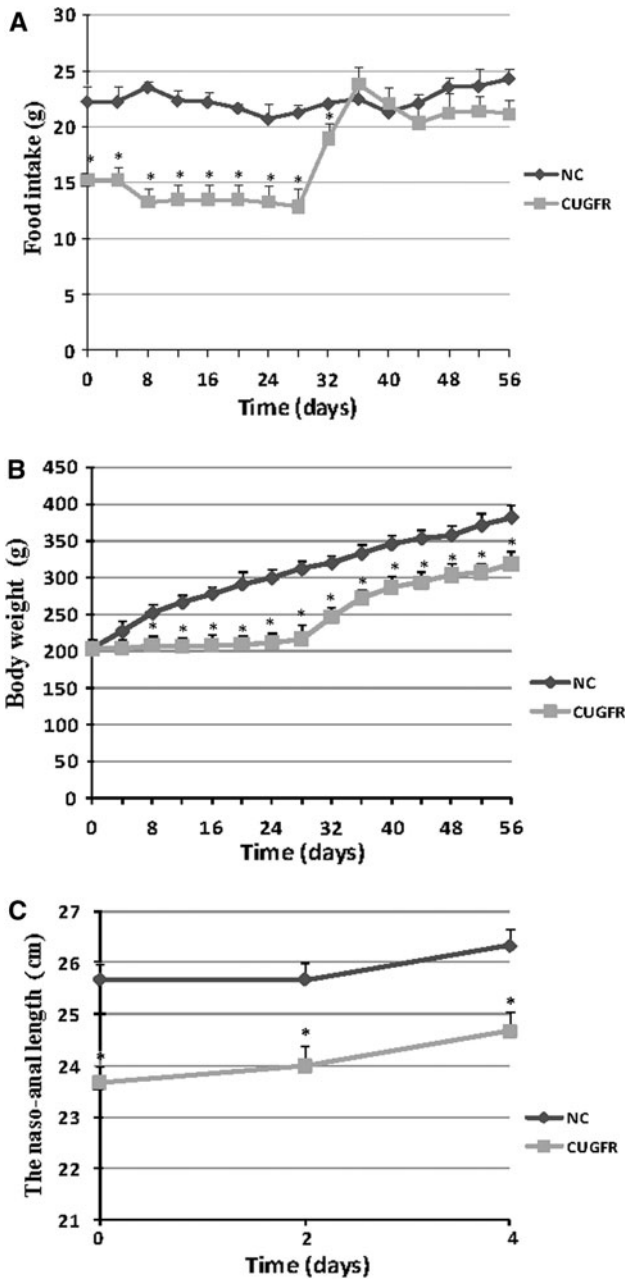

Fig. 2 Food intake (**a**), body weight (**b**), and naso-anal length (**c**) during food-restriction and re-feeding stage. All values are expressed as means ± SD ($n = 8$). *$P \leq 0.05$ versus NC group

critical adipogenesis-related gene of MSCs. Our results indicated that PPAR-gamma mRNA level was maintained at the same level as that of the control group during food-restriction period. However, it increased rapidly when catch-up growth started and was higher than that of the control group, especially when catch-up growth for 4 weeks (Fig. 8a, c2). TAZ is a main transcriptional modulator to stimulate bone development while simultaneous blocking the differentiation of mesenchymal stem cells into fat. This study showed a constant lower expression of TAZ at both protein and mRNA levels during food-restriction period. Although catch-up growth partially moderated this trend, the TAZ signals in CUGFR group failed to catch up with those of NC group even after the catch-up growth for 4 weeks (Fig. 8a, b, c3, and c4).

Discussion

Catch-up growth falls into two types in terms of age [3]. Early catch-up growth refers to children who are born small but catch up in weight and height in infancy or early childhood. Late catch-up growth affects children who may or may not be born small, but become stunted in infancy, childhood, or adulthood and catch up later in weight, becoming "stunted-obese". The latter is more commonly seen as it can happen in many cases, such as rapid economic development in developing countries, migration from rural to urban settings, war, famine, rehabilitation from chronic diseases, and so on [12, 13]. It has been suggested that late catch-up growth may be associated with the development of many metabolic diseases. In order to simulate the effects of late catch-up growth in human adults, the most prevalent form in developing countries, our study was conducted in mature rats and late catch-up growth was observed at different time points (0, 2, 4 weeks after re-feeding), with an attempt to examine the impact of catch-up growth on the body fat content and bone mineral density over time.

Obesity is a state of excess storage of body fat resulting from a chronic imbalance between energy intake and energy expenditure [14]. About 250 million adults worldwide are considered obese [15, 16]. This study showed that the individuals who undergone catch-up growth have obviously higher body fat content and further indicated the effect of catch-up growth on the distribution of body fat. That is in accordance with many studies that showed most catch-up individuals have central obesity [17]. Central obesity is an independent factor leading to metabolism syndrome [18]. So the main cause for catch-up growth leading to metabolic syndrome may be referred to central obesity. This has been confirmed in the model of intrauterine growth restriction [19].

Osteoporosis is another major public health problem, characterized by excessive skeletal fragility and susceptibility to low-trauma fracture among the elderly [20, 21]. Our study shown that there were only some bone metabolism indexes changed when catch-up growth for 4 weeks, without the morphological change of bone. This indicated that the osteoporosis resulted from catch-up growth was a gradually process. The question will arise as to when in the long run, this osteoporosis will appear. This issue is beyond

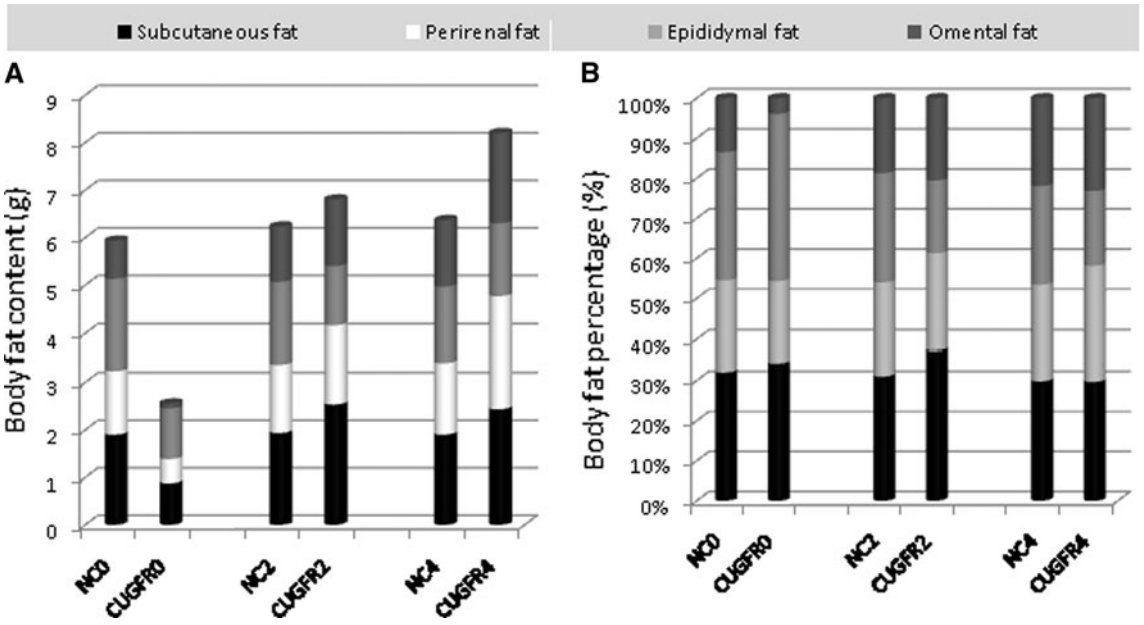

Fig. 3 Body fat content and distribution during food-restriction and re-feeding stage. White adipose tissues (subcutaneous, perirenal, epididymal, omental) were carefully dissected out and weighed immediately. The body fat content referred to the real weight of each fat. A rat's total body fat percentage is set to 100%. Body fat percentage of each adipose tissue was determined by dividing the weight of the calculated fat by the total body fat weight ($n = 8$)

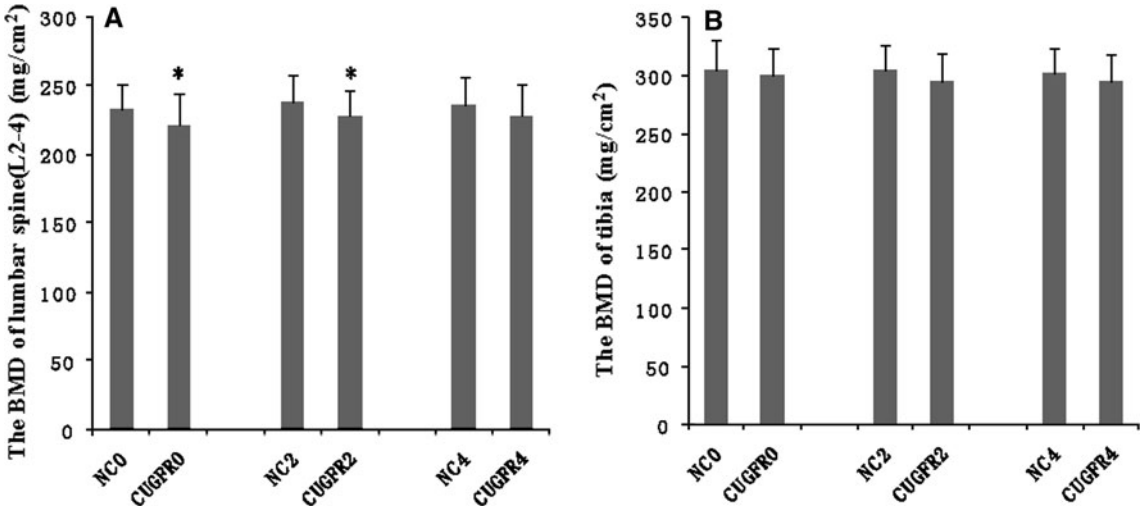

Fig. 4 Effect of catch-up growth on bone mineral density (BMD) at the levels of lumbar spine (**a**) and tibia (**b**). All values are expressed as means ± SD ($n = 8$). *$P \leq 0.05$ versus NC group

the scope of this study and will be investigated in our later studies.

Some studies demonstrated previously that catch-up growth in childhood was always associated with undesirable side effects in adult, such as osteoporosis and obesity [22]. The present study also confirmed that catch-up growth makes individuals more prone to osteoporosis and obesity. The rats which undergone catch-up growth were obviously obesity and change in some indexes about osteoporosis, such as biochemical indexes of bone metabolism and the relative genes, although histological examinations of trabecular bone had no relative change. Moreover, this indicated further the osteoporosis and obesity appeared in chronological order. In other words,

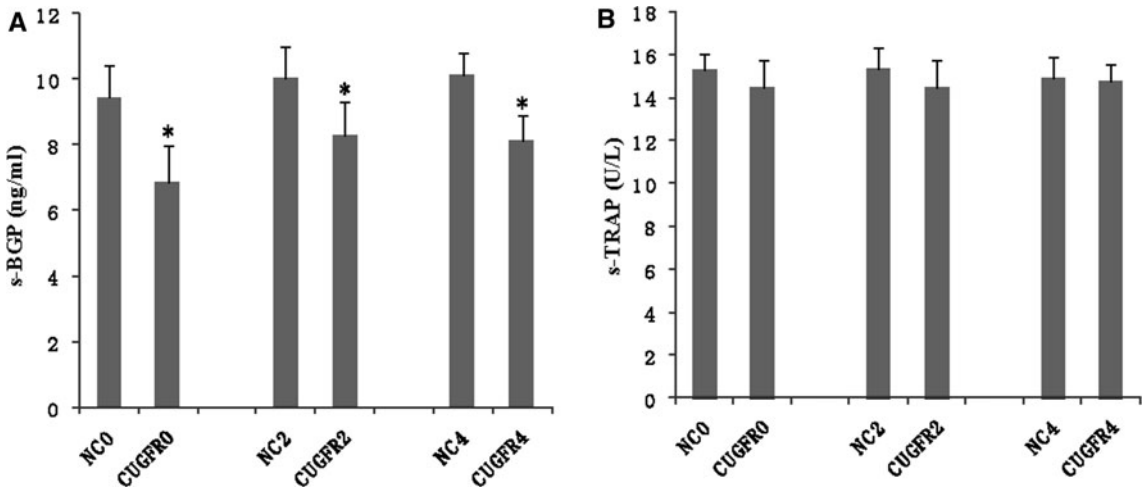

Fig. 5 Effect of catch-up growth on s-BGP (**a**) and s-TRAP (**b**). All values are expressed as means ± SD ($n = 8$). *$P \leq 0.05$ versus NC group

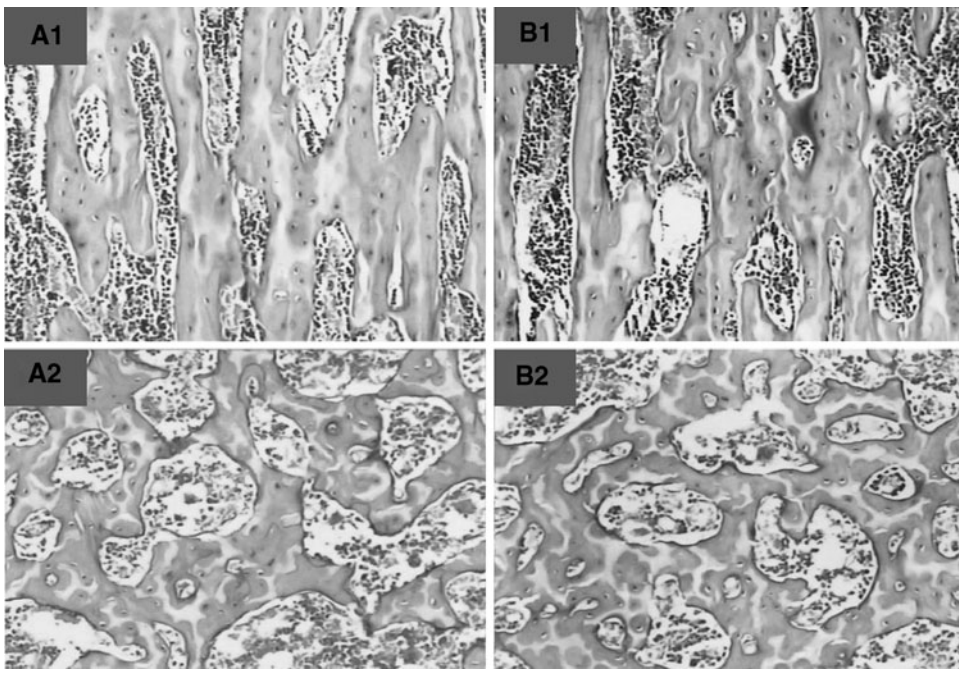

Fig. 6 Representative images showing the microstructure of trabecular bone. Trabecular bone microstructure was observed in NC4 group (**a**1, **a**2) and CUGFR4 group (**b**1, **b**2) at the vertical-sectional level (**a**1, **b**1) or cross-sectional level (**a**2, **b**2), respectively, of rat's femurs with HE staining (×100)

the catch-up individual should always be obesity first and then had osteoporosis gradually. But it is uncertain how long the interval is between the two events. Perhaps, it should be ascribed to the different metabolic rates between bone tissue and adipose tissue. Or maybe there were other factors involved in this issue, such as leptin, adiponectin, PGC-1, and so on.

MSCs are the common source of fat cells and bone cells [23]. In order to examine the detailed mechanisms through which catch-up growth leading to obesity and osteoporosis, we further isolated MSCs from bone marrow cells and detected the relative genes of MSCs differentiation. Among these genes, Runx2 is a key transcription factor associated with osteoblastic differentiation of MSCs [24, 25], and PPAR-gamma is a critical adipogenesis-related gene of MSCs [26]. Our results showed that catch-up growth led to higher level of PPAR-gamma mRNA expression but lower level of Runx2 mRNA expression. This further confirmed our hypothesis that there might be more adipogenic differentiation but

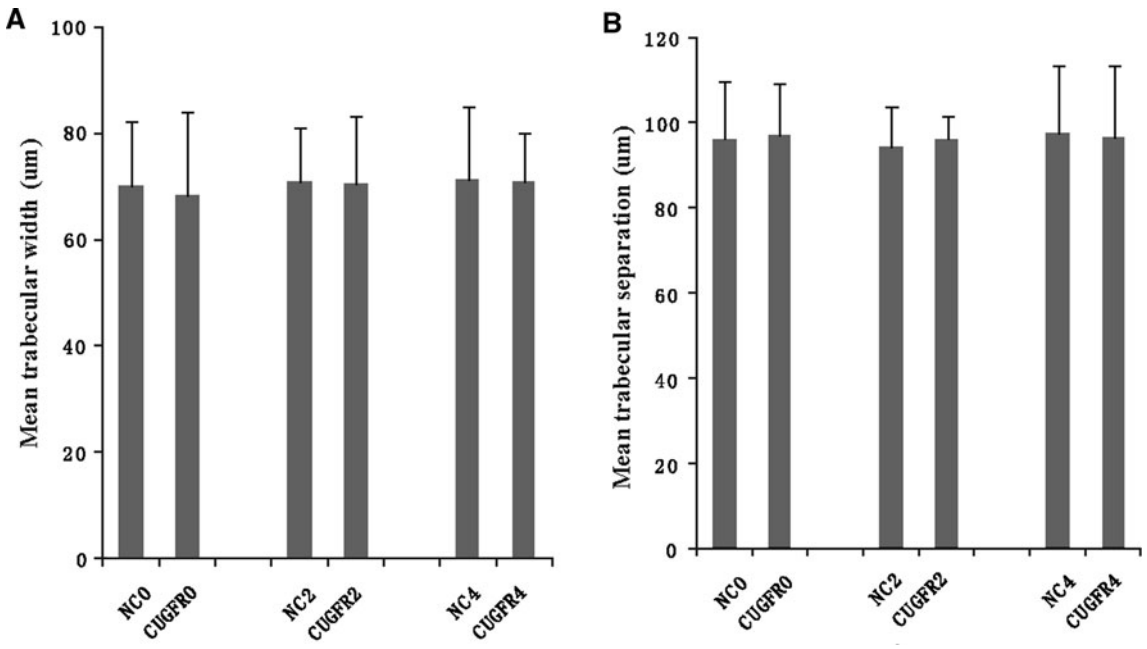

Fig. 7 Effect of catch-up growth on trabecular bone in the rat's femurs. Mean trabecular width (**a**) and mean trabecular separation (**b**) were calculated by the mean of 10 trabecular width or trabecular separation at the vertical-sectional level. All values are expressed as means ± SD

less osteoblastic differentiation of MSCs during catch-up growth. This result is in accordance with many researches [27–29], which confirmed that nutrition state could affect MSCs differentiation. It had been demonstrated that TAZ was a key gene controlling MSCs differentiation [30]. So in order to explore the further mechanisms about MSCs differential differentiation, we detected the TAZ mRNA and protein expression. The results showed that the expression of TAZ became weaken both in the mRNA and protein level with the extension of catch-up growth, indicating the controlling role of TAZ during MSCs differentiation.

From the point of thrifty gene hypothesis [31], the possible cause of MSCs differential differentiation should be speculated as below (Fig. 9). After a period of hunger, the most urgent task for one's body is to absorb more nutrition as soon as possible in order to complete two tasks, one of which is to replenish supply of nutrients and the other is to save energy for the next possible famine. As we all known, adipose tissue was generally thought to function as an energy storage reservoir, so adipogenic differentiation of MSCs is increased and thus finish the body's energy storage. Catch-up growth leads to rapid visceral fat accumulation, and the reason for this may be that the physical structure of the gut is special. The gastrointestinal tract is a site where the body exchanges energy with the external environment. As many research showed that MSCs may circulate in the blood or lymphatic flow and eventually home in different organs with the blood flow [32]. So there should be more MSCs in the intestine than other organs. When the body which is in the catch-up growth period mobilizes MSCs differential differentiation in order to regulate the body's energy storage, a lot of fat cells first appear in abdomen reasonably and thus lead to the formation of abdominal obesity. When compared with adipose tissue as an energy storage organ, bone, a supporting structure, is just a footnote to the catch-up growth individual. From the point of saving energy, short stature is more beneficial for the body to conserve energy. So it is just a response to nutritional deficiency for the body. It is a manner conducive to his own survival in the short term; however, it will result in obesity and osteoporosis in the long run.

The main limitation of our study involves its difficulty to extrapolate these results obtained in rat to humans. Moreover, like all physiological systems, the specific interactions involved in catch-up growth are far more complex. Thus, more detailed and deeper mechanism in humans should be tested in the future.

Conclusion

In brief, our experiment confirmed that catch-up growth was benefit to the adipogenic differentiation of MSCs

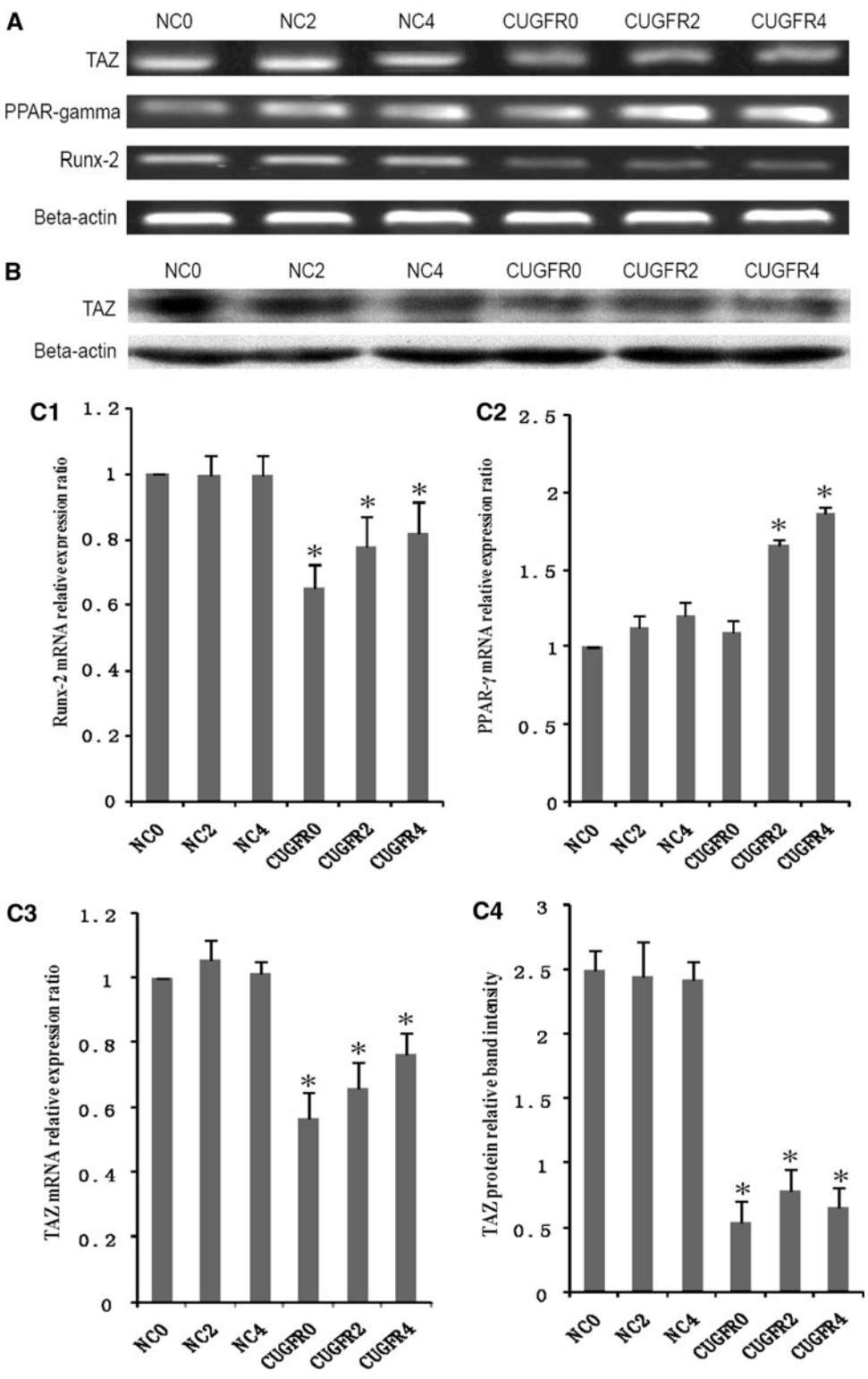

Fig. 8 Effect of catch-up growth on the adipogenic or osteoblastic differentiation-related gene expression of MSCs. **a** RT-PCR products from RNA extracted from rat ($n = 5$–6 per group) MSCs. The beta-actin control was used as control in all groups. **b** TAZ protein expression assessed by western blotting in rat MSCs. **c** The statistical results of real time quantitative PCR products (C1:Runx-2; C2:PPAR-gamma; C3:TAZ) and of western blotting products from protein (C4:TAZ) extracted from rat MSCs. Data are expressed as means ± SD. $^*P \leq 0.05$ versus NC group

and thus, suppressed the osteoblastic differentiation of MSCs, which might be responsible for the increased risk of osteoporosis and obesity during CUGFR. However, the detailed mechanisms remain unclear, and the subsequent development of osteoporosis and obesity cannot be predicted at present. Therefore, further studies are needed to establish the link between the catch-up growth-related change in the osteoporosis and obesity.

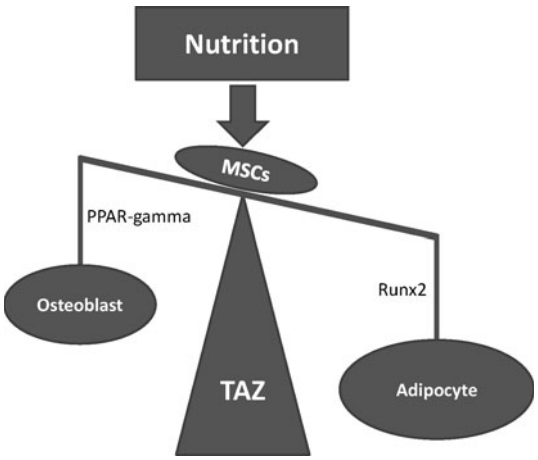

Fig. 9 Possible mechanisms of the adipogenic or osteoblastic differentiation of MSCs during catch-up growth. Nutritional rehabilitation after malnutrition could motivate catch-up growth, which probably companied with the change of MSCs differentiation. According to the modulation of the relative genes expression, MSCs may favor adipocyte differentiation and thus decrease osteoblastic differentiation during the process of catch-up growth. Among these modulation genes, TAZ plays a controlling role during MSCs differentiation

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Conflict of interest None.

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