Research Article

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New pathway of icariin-induced MSC osteogenesis: transcriptional activation of TAZ/Runx2 by PI3K/Akt

Abstract: Icariin has been demonstrated to stimulate mesenchymal stem cell (MSC) osteogenesis and activate several signals, such as PI3K/Akt, but how the osteogenesis was sequentially mediated is unclear. Runx2 is one of the osteogenic regulators in MSC and is regulated by the TAZ gene. The purpose of this study was to investigate whether icariin-activated PI3K/Akt crosstalked with the TAZ-Runx2 pathway to regulate MSC osteogenesis. Adipose-derived MSCs were treated with icariin alone, together with TAZ silencing or PI3K/Akt inhibitor. Normal MSCs were used as a control. The activation of PI3K/Akt, expression of TAZ and downstream expression of Runx2 were analyzed. Induction of MSC osteogenesis under different treatments was detected. The results demonstrated that icariin treatment significantly activated PI3K/Akt and TAZ expression, as well as the downstream Runx2 expression. When activation of PI3K/Akt by icariin was inhibited by LY294002, upregulated TAZ expression was reversed, as well as the downstream expression of Runx2. Consequently, with the osteogenic counteracting effects of icariin on MSCs, inhibition of TAZ upregulation by siRNA did not significantly influence PI3K/Akt activation in icariin-treated MSCs, but icariin-induced upregulation of Runx2 and osteogenic differentiation in MSCs was counteracted. It could be concluded from these findings that icariin treatment activated PI3K/Akt and further mediated the transcriptional activation of the TAZ/Runx2 pathway to induce osteogenic differentiation of MSCs.

Keywords: Icariin, PI3K/Akt, TAZ/Runx, MSCs, Osteogenic differentiation

1 Introduction

Diseases associated with osseous defects, such as osteoporosis and bone fractures, are becoming increasingly prevalent due to the increased aging population and trauma events. Though many techniques have been developed in past years, the treatment of serious bone defects remains a great challenge in orthopedic surgery [1, 2]. In recent years, development in the field of stem cells and tissue engineering has proposed a novel therapeutic strategy. Mesenchymal stem cells (MSCs) have the potential to differentiate into osteoblasts. Moreover, MSCs are abundantly distributed in adult tissues and show a low immunogenicity. Therefore, they have become the most commonly used seeding cells in bone regeneration and have attracted increasing attention [3-5]. In bone tissue engineering and clinical practice, enhancing the differentiation of seeding cells is important for effective repair of bone defects. Accordingly, numerous growth factors (GFs) and/or drugs have been applied to induce osteogenic differentiation from MSCs, such as bone morphogenetic proteins (BMPs), basic fibroblast growth factor (bFGF) and simvastatin, etc [6-8]. In comparison, GFs showed potent effects on the proliferation and osteogenic differentiation of MSCs. However, GFs were usually expensive which would result in a high cost. Moreover, GFs were usually of short half-life and easily degraded. These problems have limited their widespread usage [9]. Thus, osteogenic drugs which were of lower costs and longer efficacies than GFs should be a more suitable option in clinical practice in the future.

Icariin, used in Chinese medicine, has been confirmed to have a potent capacity to induce MSC differentiation into
 osteoblasts by independent research groups [10, 11]. The drug was determined clinically safe and approved for use in drug preparations [1, 11, 12]. The application of icariin to enhance osteogenic differentiation of MSCs was promising. Previously, several studies have explored the mechanisms of icariin-induced osteogenic differentiation [1, 10, 13]. In a recent report, a novel mechanism has been revealed in which PI3K/Akt and its downstream pathway played a crucial role in osteogenic differentiation of MSCs induced by icariin [11]. However, the underlying mechanisms were far from thoroughly revealed. Moreover, it was known, that osteogenesis of MSCs was regulated by a series of regulators and signal molecules, such as transcriptional coactivator with PDZ-binding motif (TAZ), BMP signals, and Peroxisome proliferator-activated receptor gamma (PPARγ) [14, 15]. However, which osteogenic regulator has crosstalk with icariin-activated PI3K/Akt was unknown.

TAZ played a vital role in the osteogenic differentiation of mesenchymal stem cells (MSCs), and strongly activated runt related transcription factor 2 (RUNX2)-driven genes during the terminal osteogenic differentiation [16]. It has been demonstrated that TAZ-Runx2 is a key pathway through which an inducer promoted MSC osteogenesis [16]. In the present study, we aimed to investigate whether icariin-activated PI3K/Akt would further crosstalk with the TAZ-Runx2 pathway to promote osteogenic differentiation in MSCs. The activation of PI3K/Akt, expression of TAZ and downstream expression of Runx2 was analyzed. Finally, the osteogenic capacities of MSCs under different treatments were detected.

2 Materials and methods

Icariin was purchased from Tauto Biotech (Shanghai, China); LY294002 was purchased from Sigma(USA).

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

2.1 Isolation and characterization of rat adipose-derived mesenchymal stem cells

Inguinal adipose tissues were obtained from 2 weeks old rats (The main reason for choosing 2 weeks old rat for MSC preparation was that stem cells were more abundant and have a better self-renewal capacity in young rats than in adult ones). Isolation of adipose-derived MSCs was performed according to the previously reported protocol [3, 17, 18]. Briefly, the adipose tissues were washed three times with aseptic phosphate buffer solution (PBS). After the contaminating debris and red blood cells were removed, the tissues were cut into no more than 1 mm³ size pieces and 0.1% collagenase I (Sigma) was added to digest the tissues for one hour. Then, equal volume of Alpha modified minimum essential medium (αMEM, Gibco)/10% fetal bovine serum (FBS,Gibco) was added to terminate the digestion. The digested solution was filtered through 80μm mesh and transferred into a 10M centrifuge tube. After centrifugation for 10min at 800g, the supernatant was removed and the cell pellet was resuspended in fresh α-MEM containing 10% FBS. The cells were counted and seeded onto culture flask. The flasks were put into a 37°C, 5% CO₂ incubator. The culture medium was changed every two days. When 90% confluence was reached, cells were collected by digestion with 0.25% trypsin and passaged at the ratio of 1:3.

After three passages, MSCs were collected and immunostained with FITC-conjugated antibodies against CD90, CD105, CD45, CD34 and CD31. Then, the immunophenotype was analyzed by flow cytometry. To determine the multipotency of MSCs, the cells were cultured in adipogenic or osteogenic medium to induce their differentiation. The osteogenic and adipogenic mediums were the same as the previous report [17]. After 2 weeks, adipogenic differentiation was detected by oil red O staining; after 3 weeks, osteogenic differentiation was detected by alizarin red staining.

2.2 MSC treatment with icariin

To assay cytotoxicity of icariin, MSCs were seeded onto 96-well plates and allowed to grow to 80% confluence. Then, the doses of 100, 50, 10, 1, 0.1, 0.01 μM icariin were added into cell culture and incubated for 24h (n=5 for each condition). Normal cultured cells without icariin were used as control. Subsequently, cells were collected by trypsin digestion. MSCs were stained with propidium iodide (PI) solution. Dead cells were counted by flow cytometry. The cell viability was expressed as percentage of viable cells.

2.3 Optimization of icariin concentration for MSCs differentiation

To determine the optimal concentration of icariin for osteogenic differentiation of MSCs, 0.01, 0.1, 1, 10, 50 and 100 μM icariin were supplemented into culture medium
(n=5 for each condition) as previously reported [1, 11]. MSCs were seeded into 24-well plates and cultured in icariin-containing medium. Normal cultured MSCs without icariin were used as control. The medium was changed every two days. Osteogenic differentiation marker, alkaline phosphatase (ALP) was measured after 10 days' differentiation. Osteocalcin and calcium deposition were also detected at 2 weeks. The optimal icariin concentration for MSCs differentiation was determined by comparing the osteogenic differentiation at different concentrations.

2.4 RNA interference

Small interfering RNAs targeting TAZ mRNA (SiTAZ) were purchased from Genepharma Corporation (China). 20 nM siRNAs were used for cell transfection with lipofectamine RNAiMAX reagent (Genepharma Corporation, China) according to the manufacturer’s instruction. Previously, it has been demonstrated that efficient RNAi occurred within 24h after liposome-based siRNA delivery and the potent RNAi occurred between 2-7 days. Then, the targeted gene will gradually recover, but the significant lower expression could last for about 2 weeks or even longer before it recovers to normal levels [19]. Therefore, in each experiment involving the RNAi, siRNA was delivered 24h before the addition of chemicals.

The sequences of SiTAZ were: 5'-GGCCAGAGAUAUUUCCUATT-3' and 5'-UAAGGAAAUAUCUCUGGCTT-3'.

2.5 RT-PCR

Cells were collected by trypsin digestion. Trizol was used for cell lysis and then, RNAprep pure Cell/Bacteria Kit (Qiagen) was used for total RNA extraction and purification. Advantage RT-for-PCR Kit was used for reverse transcription.

Then, QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used in the following PCR procedure. Each sample was replicated three times. The relative expression of target genes was quantified as previously reported [11, 12]. The gene-specific primers for PPARγ, Runx2, OCN, BSP and ALP in the study were designed using primer3.0.

2.6 Western Blotting

After 48h treatment under different conditions, cells were collected and lysed in Laemmli sample buffer (Bio-Rad). Total proteins were extracted and the concentrations were determined using the BCA™ Protein Assay Kit (Thermo Scientific). For electrophoresis analysis, about 60 μg of protein was loaded onto 15% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF membrane (Roche). Then, the PVDF membrane was blocked with 5% nonfat dried milk (in TBST) for 1h. The PVDF membrane was incubated with primary antibodies overnight at 4°C. TAZ (Abcam, 1:1000), p-Akt (Abcam, 1:5000), Akt (Abcam, 1:500) and GAPDH (Abcam, 1:5000) were detected respectively. After washing with TBST three times (10 min for each time), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, 1:1000) at room temperature for one hour. The protein bands were detected using enhanced chemiluminescence reagent (Applygen). Each sample was repeated 5 times.

2.7 Measurement of ALP activity

ALP activity was measured in accordance to the previous report [11]. Cells were collected and centrifuged. Lysis buffer was added and freezing/thawing processes were performed to disrupt the cell plates. Subsequently, ALP reaction media was added for enzymatic reaction. A commercial DC protein assay kit (BioRad) was used to determine the total protein content. The sample was then measured using a spectrophotometer at 405 nm and APL activity was counted. Each sample was replicated for at least three times.

2.8 Determination of osteocalcin and calcium deposition levels

Osteocalcin in culture medium was determined by enzyme immunoassay (ELISA) using a commercial osteocalcin Kit (Immunodiagnostic Systems Ltd., Boldon, UK). The calcium deposition was measured as previously reported [11, 16]. The cells were washed twice with PBS. Then, 1 ml of 1M HCl was added and incubated overnight with gentle shaking. A commercial O-cresolphthalain complex method with Calcium Colorimetric Assay Kit was used to measure the released Ca2+.
2.9 Statistical analysis

All data were expressed as mean±SD. SPSS 17.0 software was employed for statistical analyses. Student t-test was performed for comparison between different groups. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Culture and Characteristics of MSCs

In culture flask under normal conditions (37°C, 5% CO$_2$), MSCs demonstrated fibroblast-like morphology. Flowcytometry analysis showed that most MSCs expressed CD105 and CD90, yet did not express CD45, CD31 and CD34. After culture in differentiation medium, adipogenic and osteogenic capacities were confirmed by oil red O staining and alizarin red staining, respectively (Figure 1).

3.2 The optimal dose of icariin

PI staining and flow cytometry analysis showed that low concentrations of icariin (0.01, 0.1 and 1 μM) did not influence the viability of MSCs compared with normal conditions. When the icariin concentration was increased to 10 μM, the cell viability was significantly decreased ($p<0.05$), but the cell viability was still above 90%. Increasing the concentration of icariin to 50 μM significantly reduced cell viability below 90% (Figure 2A). To determine the optimal dose of icariin to induce osteogenic differentiation, MSCs were cultured in icariin-containing medium. Measurement of ALP at 10 days demonstrated that 10 μM was optimal to induce osteogenic differentiation from MSCs compared with the other doses (Figure 2B), while MSC viability was maintained above 90%. Osteocalcin and calcium deposition at 2 weeks was consistent with ALP measurement, so that the optimal osteogenic differentiation was detected at 1-10 μM icariin (Figure 2C and D).

3.3 PI3K/Akt activation mediated the upregulation of TZA-Runx2 pathway in icariin-treated MSCs

It was demonstrated that 10 μM icariin treatment significantly upregulated the expression of p-Akt and increased the expression of TAZ compared with control.

![Figure 1. Cultivation and characterization of MSCs. A, Representative images of flowcytometry analysis; B, Quantitative analysis of MSC immunophenotype from flowcytometry data; C, Adipogenic differentiation of MSCs was detected by oil red O staining, while osteogenic differentiation of MSCs was detected by alizarin red staining.](image-url)
demonstrates that direct intervention of TAZ by RNAi was similar to PI3K/Akt intervention in counteracting the upregulation of TAZ by icariin.

Icariin treatment significantly upregulated Runx2 expression, but the upregulation was counteracted by TAZ inhibition and PI3K/Akt inhibition (Figure 3E). We also demonstrated that the expression of PPARγ was consistent with TAZ-Runx2 expression (Figure 3F). From the above data, we show that icariin treatment activated PI3K/Akt inhibition which subsequently activated the TAZ-Runx2 pathway for MSC osteogenesis.

3.4 Icariin induced osteogenetic differentiation from MSCs via PI3K/Akt and TZA-Runx2 pathway

As shown in Figure 4, the osteogenic related genes Runx2, OCN, BSP and ALP were comparable among different groups at day0. At day7, icariin treatment significantly increased the expression of osteogenic related genes. When icariin treatment was performed together with LY294002, upregulated osteogenetic makers by icariin were significantly neutralized. Such neutralization of the effects of icariin was also achieved by TAZ inhibition. As shown in Figure 5A, significantly more positive stained calcified tubercles were observed than the control. TAZ silencing significantly reduced Alizarin red-stained
Figure 3. PI3K/Akt-TAZ/Runx2 activation and the expression of related osteogenic regulator. A, Icariin upregulated both PI3K/Akt and TAZ/Runx2 pathways, but their interaction was unknown (a), inhibition of PI3K/Akt and TAZ/Runx2 respectively was designed to verify the potential interaction of the pathways (b); B, TAZ silencing at different doses of siRNA (siTAZ indicates siRNA targeting TAZ; siCon indicates negative control); C, p-Akt expression in icariin-treated MSCs with the presence of TAZ silencing or LY294002 (PI3K inhibitor); D, TAZ expression in icariin-treated MSCs with the presence of TAZ silencing or LY294002; E, Runx2 expression in icariin-treated MSCs with the presence of TAZ silencing or LY294002; F, PPARγ expression in icariin-treated MSCs with the presence of TAZ silencing or LY294002 (*P<0.01 compared with control).

Figure 4. RT-PCR analysis of osteogenic related genes. Expression of osteogenic genes of A, Runx2; B, OCN; C, BSP and D, ALP in MSCs treated by icariin in the presence of TAZ silencing or PI3K inhibitor (**P<0.01 compared with control; ##P<0.01 compared with icariin+TAZ silencing; &P<0.01 compared with icariin+ LY294002 treatment).
G. Zhang, et al. have focused on the mechanism of icariin-induced differentiation of MSCs and different mechanisms were revealed by independent groups. Zhai et al. demonstrated that the PI3K/Akt pathway was an important pathway that was activated in icariin-treated MSCs [11]. Further, they found that several signal molecules downstream of PI3K/Akt, such as eNOS and NO, may play important roles in the differentiation of MSCs. Wei et al. found that ERα-Wnt/β-catenin signaling was another pathway involved in the icariin-stimulated osteogenic differentiation of MSCs [13]. The most recent study by Ye et al. found that the icariin-induced MSC differentiation was also related to the transcriptional activation of TAZ signaling [10].

The various signaling pathways revealed by these studies indicate the complicated network underlying icariin-induced osteogenic differentiation of MSCs. However, whether crosstalk between different signaling pathways existed, such as PI3K/Akt and other pathways, was unclear. In the present study, we demonstrated that TAZ-Runx2, an important pathway in osteogenic differentiation, was significantly upregulated in icariin-treated MSCs accompanying the activation of the PI3K/Akt pathway. More importantly, we found that upregulated TAZ-Runx2

calcified tubercles, which was similar to what was observed for LY294002. In addition, we also detected ALP activity, calcium salt deposition and osteocalcin content. The results were consistent with the above observation, that TAZ silencing inhibited osteogenic differentiation of icariin-treated MSCs and this was similar to the effect produced by LY294002 treatment (Figure 5B).

Collectively, based on the previous report about the role of PI3K/Akt in icariin-induced osteogenic differentiation from MSCs, the above results indicated that PI3K/Akt mediated osteogenic differentiation of icariin-treated MSCs was further mediated through crosstalk with the TAZ-Runx2 pathway.

4 Discussion

Icariin is used in Chinese medicine and is extracted from Epimedium. Independent studies have previously confirmed that icariin is a potent inducer for osteogenic differentiation of MSCs [1, 11, 12]. As a promising drug to facilitate MSC-based bone regeneration, the mechanism of icariin-induced osteogenic differentiation attracted significant attention. Recently, several studies have focused on the mechanism of icariin-induced differentiation of MSCs and different mechanisms were revealed by independent groups. Zhai et al. demonstrated that the PI3K/Akt pathway was an important pathway that was activated in icariin-treated MSCs [11]. Further, they found that several signal molecules downstream of PI3K/Akt, such as eNOS and NO, may play important roles in the differentiation of MSCs. Wei et al. found that ERα-Wnt/β-catenin signaling was another pathway involved in the icariin-stimulated osteogenic differentiation of MSCs [13]. The most recent study by Ye et al. found that the icariin-induced MSC differentiation was also related to the transcriptional activation of TAZ signaling [10]. The various signaling pathways revealed by these studies indicate the complicated network underlying icariin-induced osteogenic differentiation of MSCs. However, whether crosstalk between different signaling pathways existed, such as PI3K/Akt and other pathways, was unclear.

In the present study, we demonstrated that TAZ-Runx2, an important pathway in osteogenic differentiation, was significantly upregulated in icariin-treated MSCs accompanying the activation of the PI3K/Akt pathway. More importantly, we found that upregulated TAZ-Runx2
expression by icariin could be counteracted by PI3K/Akt inhibitor, which further inhibited the icariin-induced osteogenic differentiation of MSCs. However, inhibition of TAZ by RNAi did not influence the activation of PI3K/Akt.

Currently, no report has shown that PI3K/Akt was a direct regulator for MSC osteogenic differentiation. Therefore, it could be inferred that activated PI3K/Akt by icariin may crosstalk with a downstream pathway which would further regulate osteogenic differentiation. TAZ is an important regulator in osteogenic differentiation of MSCs [20]. It has been demonstrated that upregulated TAZ would strongly activate the downstream factor Runx2 to promote osteogenic differentiation [16]. Previously, several inducers have been demonstrated to promote osteogenesis directly through TAZ activation, or some other pathway-mediated TAZ activation, such as IGF1, FGF2 and lysophosphatidic acid (LPA) [21-23]. Crosstalk between other pathways and the TAZ pathway was also demonstrated during osteogenic differentiation of MSCs [21, 24]. A typical case was reported in FGF2 induced differentiation from osteoblasts [21]. It was found that FGF2 treatment firstly activated ERK and then, activation of ERK would upregulate TAZ expression and facilitate its interaction with downstream Runx2 [21]. More significantly, a recent report found that low-power laser irradiation (LPLI) activated the Akt/GSK3β pathway which further activated TAZ-related pathway to stimulate osteogenesis [25]. These reports suggest that TAZ may be a key effector of many drugs to promote osteogenesis and furthermore, crosstalking with the TAZ pathway may be the underlying mechanism by which some indirect signal molecules regulate osteogenesis, such as ERK and Akt/GSK3β. In the present study, we found that icariin activated PI3K/Akt would further crosstalk with the TAZ pathway to promote osteogenesis of MSCs, providing a novel mechanism of icariin-induced osteogenic differentiation of MSCs.

The activation of TAZ or related pathway by icariin has been reported by independent studies during osteogenesis. Zhao et al. found that in osteoblasts (in vitro) icariin-induced osteogenic differentiation was Runx2-dependent [1]. Recently, Ye et al. showed that icariin promoted proliferation and osteogenic differentiation of adipose-derived MSCs through activation of the RhoA-TAZ signaling pathway [10]. Furthermore, it was suggested that the icariin-activated PI3K/Akt pathway may be correlated with TAZ-related pathway during MSC osteogenesis. In the present study, we provide direct evidence that crosstalking between PI3K/Akt and TAZ-Runx2 pathways exists and regulates MSC differentiation under icariin treatment. More importantly, it was revealed that TAZ-Runx2 activation during icariin treatment was mediated by PI3K/Akt, so that TAZ-Runx2 was downstream of the PI3K/Akt pathway.

It was worth noting that transcription, translocation and phosphorylation of TAZ are three important aspects for its function and gene regulation. All three aspects were reported respectively to influence the differentiation of MSCs under some conditions [20, 25, 26]. In the present study, the main purpose was to investigate whether icariin-activated PI3K/Akt crosstalked with TAZ-Runx2 pathway from a transcriptional level and further regulated MSC osteogenesis, not translocation or phosphorylation of TAZ. Of course, the role of TAZ translocation or phosphorylation may be related to the icariin-induced MSC differentiation and deserves in-depth investigation, which will be primarily considered in our next work.

In conclusion, this study demonstrated crosstalking between the PI3K/Akt and TAZ-Runx2 pathway in icariin-treated MSCs. That was, icariin treatment activated the PI3K/Akt pathway and subsequently, activated PI3K/Akt would upregulate TAZ and the downstream effector Runx2 which regulated the differentiation of MSCs towards osteogenic lineage. Collectively, it could be concluded that icariin stimulated osteogenic differentiation of MSCs through the PI3K/Akt-TAZ-Runx2 pathway. These findings provide novel insight into the mechanisms of icariin-induced osteogenic differentiation of MSCs.

Conflict of interest: Authors state no conflict of interest

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