Retinoic Acid Promotes Retinoic Acid Signaling by Suppression of Pitx1 In Tendon Cells: A Possible Mechanism of a Clubfoot-Like Phenotype Induced by Retinoic Acid

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Background: The pathogenesis of idiopathic congenital clubfoot (CCF) is unknown. Although some familial patients have Pitx1 mutations, and the Pitx1+/− genotype causes a clubfoot-like phenotype in mice, the mechanism of Pitx1-induced CCF is unknown.

Material/Methods: We used tibialis anterior tendon samples to detect the expression of Pitx1 in idiopathic and neurogenic clubfoot patients. After obtaining Sprague-Dawley (SD) rat Achilles tendon cells, the expression of Pitx1 was knocked down by SiRNA. After 48 h of culture, mass spectrometry was used to quantitatively analyze proteins. Then, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to assess the downstream pathway of PITX1. The relationship between Pitx1 and the promoter region of deacetylase 1 (Sirtuin-1 and Sirt1) was examined by luciferase and ChIP assays.

Results: We found that Pitx1 expression in the tendon samples of idiopathic CCF patients was downregulated. Mass spectrometry analysis revealed that the inhibition of Pitx1 induced the downregulation of Sirt1 expression in tendon cells. Luciferase and ChIP assays confirmed that Pitx1 binds to the promoter region of SIRT1 and promotes Sirt1 gene transcription. Further results showed that, after the inhibition of Pitx1 in tendon cells, CRABP2 acetylation increased, the nuclear import of CRABP2 was enhanced, and the expression of RARβ2 increased. After the inhibition of Pitx1, RARβ2 expression was further increased by RA treatment in tendon cells. In the presence of retinoic acid, the expression of Pitx1 was inhibited in tendon cells.

Conclusions: Pitx1 binds to the promoter region of SIRT1 and promotes the transcription of SIRT1. Positive feedback occurs between RA signaling and Pitx1.

MeSH Keywords: Achilles Tendon • Clubfoot • Sirtuin 1

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**Background**

Congenital clubfoot (CCF), or congenital talipes equinovarus (CTEV), is one of the most common congenital foot deformities and occurs at about 1 out of 1000 neonates [1–3]. Clubfoot is characterized by structural defects of several tissues of the foot and lower leg, which leads to abnormal positioning of foot and ankle joints [4,5]. The Ponseti method has been shown to be the most effective treatment for CCF [2]. Now, the Ponseti method has become the most widely used treatment for CCF [6].

The cause of CCF is still unclear, but some possible pathogenic genes have been reported, such as PITX1, TBX4, and Homeobox A and D [7–9]. Pitx1 is a bicoid-related homeodomain transcription factor that was first identified in a genome-wide linkage study (5q31: LOD: 3.31). Pitx1 determines the morphology of muscle, tendon, and bones of the hindlimb [10]. Interestingly, the E130K mutation of Pitx1 (c.388G/A) suppressed wild-type Pitx1 activity in a dose-dependent manner in 9 affected individuals from a 5-generation family [7]. Additionally, research showed that a clubfoot-like phenotype was found in 20 of 225 Pitx1(+/–) mice [3,11,12]. These deformities include forefoot cavus in the frontal plane and hindfoot equinus in the sagittal plane (short Achilles tendon) and are associated with abnormalities of vascular, fibular bone volumes, and muscle volume in mice with the clubfoot-like phenotype. The authors suggested decreased Pitx1 activity due to the E130K/Pitx1 mutation as a cause for CCF and cited support based on a previous study [7]. However, most clinical CCF cases do not involve familial inheritance or the Pitx1 gene mutation [7]. A decrease in Pitx1 expression in patients with nonfamilial clubfoot has not been reported, and considerations of medical ethics have limited access to specimens for such studies.

In the Ponseti Center of Excellence, 3 idiopathic CCF patients and 2 patients with neurogenic clubfoot requiring tibialis anterior tendon transfer (TATT) surgery signed an informed consent form and agreed to the use of the abandoned tendon tissue as a study sample. Our small case study was performed to determine if there was a decrease in Pitx1 expression in the tibialis anterior tendon from the 3 idiopathic CCF patients. However, the mechanism of the downregulation of Pitx1 expression involved in clubfoot deformity is not clear. The purpose of this study was to analyze the downstream signals of Pitx1 to explore the possible pathogenesis of Pitx1.

**Material and Methods**

**Procurement of human tendon tissue**

Human tendon tissue was obtained from 5 patients (5–8 years old) who required anterior tibialis tendon transfer surgery. There were 3 idiopathic CCF patients and 2 patients with neurogenic clubfoot. After birth, the 3 idiopathic CCF patients (2 male and 1 female) had been diagnosed with idiopathic CCF and were treated with the Ponseti method. After the end of treatment, at the age of 5 years, relapses occurred and were treated with the Ponseti method. Two patients (2 males) with neurogenic clubfoot with normal feet at birth developed neurological symptoms with growth, followed by foot deformities. They were diagnosed with clubfoot secondary to tethered cord syndrome (TCS), then were treated with the Ponseti method. TATT was performed for gait. All tissues were obtained with full informed consent and prior institutional ethics committee approval (the Ethics Committee at Xin Hua Hospital, School of Medicine, Shanghai Jiao Tong University, approval no. XHEC-D-2019-40). All samples were harvested from abandoned tendon tissue. The tendon tissues were fixed with 4% paraformaldehyde and then used to assess the expression and distribution of Pitx1 by immunohistochemistry.

**Harvesting and culturing of tendon cells**

Newborn SD rats (7 days old, male) were used for dissection of the Achilles tendon. Tendon cell isolation was performed as previously described [13]. Cells suspensions were cultured in DMEM supplemented with 10% FBS at 5% CO2, [14]. We used cells at passages 2–3 for all experiments.

**Antibodies and reagents**

For Western blotting, we used the following antibodies: Pitx1 (Abcam: ab172117 [dilution 1: 500]), Sirt1 (Abcam: ab189494 [dilution 1: 500]), Sirt6 (CST: 12486 [dilution 1: 500]), Sirt7 (CST: 5360 [dilution 1: 500]), CRABP2 (Abcam: ab211927 [dilution 1: 500]), RARβ2 (Abcam: ab151570 [dilution 1: 1000]), and GAPDH (Abcam: ab181602 [dilution 1: 2000]). For immunohistochemical testing, we used anti-Pitx1 (Abcam: ab172117 [dilution 1: 150]). For immunofluorescence analysis, we used an antibody against CRABP2 (Abcam: ab211927 [dilution 1: 200]). For chromatin immunoprecipitation (ChIP) assays, we used 4 μg of an antibody against Pitx1 (Abcam: ab172117 [dilution 1: 100]). All procedures were performed according to the manufacturer’s instructions.

**Gene knockdown using small interfering RNA (siRNA)**

To silence Pitx1 expression in tendon cells, cells were transfected with siRNA using RNAiMAX transfection reagent (Invitrogen).
siRNA-A and siRNA-B were synthesized by Shanghai Genechem Co. with TD and the recognized sequences “gcuguggagugc-guacguug” and “uguguacugcgcggugua”, respectively. The silencing effects on Pitx1 were confirmed by Western blot after 48 h of transfection.

**Immunohistochemical analysis**

Tendon tissue were obtained from 3 patients with CCF and 2 patients with neurological CCF and were subjected to IHC analysis using anti-Pitx1 antibodies. Briefly, the tendon was de-paraffinized and rehydrated and then subjected to antigen retrieval by incubating the tissues in hot (95°C) sodium citrate buffer (0.01 M, pH 6.0) for 10 min. The tissue sections were exposed to hydrogen peroxide (3% H₂O₂) for 5 min to quench the endogenous peroxidase and then blocked in 30% horse serum for 30 min. The slides were incubated at 4°C with primary Pitx1 antibodies (1: 150 dilution) for 14 h. After the tissues were washed with 1×TBST (Tris-buffered saline containing 0.1% Tween-20), the slides were then incubated with biotinylated secondary antibodies (anti-Rabbit IgG; Abcam) and detected using an ABC kit (Beyotime Biotechnology, China).

**Immunofluorescence**

To immunostain cells, the samples were fixed in 4% paraformaldehyde in PBS for 40 min at room temperature. After the cells were washed 3 times in PBS/0.1% BSA for 5 min, they were permeabilized using 0.2% Triton (Sigma, St Louis, MO, USA; T9284) in PBS for 20 min and then washed in PBS/0.1% BSA. Primary antibodies against CRABP2 (Abcam, Cambridge, UK) were diluted in PBS/0.1% BSA at 4°C. After washing, the cells were incubated with a FITC-conjugated goat anti-rabbit secondary antibody (1: 500; Abcam) and DAPI (Sigma) for 1 h at 30°C. Fluorescent images were obtained using a Nikon microscope.

**Mass spectrometry**

Pitx1 knockdown rat tendon cells and control cells were collected and lysed. The protein samples were assayed by iTRAQ® mass method. The standard of proteins expression difference was fold change <0.8 or >1.2. Then, the identified differentially expressed proteins were analyzed by GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis (http://david.abcc.ncifcrf.gov).

**Luciferase reporter assay**

The Sirt1 promoter area (–2000 to 1) was cloned to pGL3-basic plasmid (Promega, Madison, WI, USA). The pGL3-Sirt1-pr was co-transfected with Renilla luciferase and Pitx1 overexpression plasmid into tendon cells using lipofectamine (Invitrogen), then after 24-h transfection, cell lysates were analyzed by the Dual Luciferase Assay system (Promega) as described previously [15].

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assay was carried out as previously reported [15] at site 1 (–1185 1079 to –103084) and at site 2 (–2659 to –2654). The primers used were GCAAGGCAAGCAGTTTGTAAA CTGCCATGTTCTATCCACTGCT-3’, 5’-TTCAATCACTGCTAAGT GCCA-3’ and 5’-GAGGATCCGCCATGTCTC-3’. The 2 amplicons were 156bp and 189bp, separately. CRABP2 protein purification rat tendon cells were lysed, and the lysis supernatant was incubated with a CRABP2 antibody and protein A beads overnight at 4°C. The protein A beads were washed 3 times with RIPA buffer and then boiled with 1×SDS loading buffer [15]. The boiled protein sample was separated by SDS-PAGE. CRABP2 protein bands were detected by anti-acetyl lysine and analyzed.

**Statistical analysis**

All experiments were repeated 3 times. Differences between 2 groups were analyzed using the paired-sample t test with Prism 5.0 software (GraphPad, La Jolla, CA). Differences among at least 3 groups were analyzed using one-way ANOVA with SPSS v20.0 for Windows (IBM, Armonk, NY). P<0.05 was considered significant.

**Results**

**Reduced expression of Pitx1 in the anterior tibialis tendon in idiopathic CCF.**

Although the PITX1 gene mutation was not found in most idiopathic CCF patients, it is unclear whether Pitx1 expression is decreased in human CCF patients [7,11]. With the increased use of the Ponseti method, it is very difficult to obtain relevant tissue specimens; this also limits understanding of the etiology of CFF. Some patients with recurrent CCF or neurogenic CCF require anterior tibialis anterior muscle transfer to improve gait. We obtained the relevant specimens after approval by the Ethics Committee and the patients’ families. The pathogenesis of neurogenic clubfoot is clearly caused by neuromuscular abnormalities, such as those in patients with tethered cord syndrome (TCS). In addition, samples from patients with neurogenic clubfoot can be used as a control for idiopathic CCF samples. Our small case study showed that, in 3 idiopathic CCF patients, there was a decrease in Pitx1 expression in the anterior tibialis tendon compared with that in 2 patients with clubfoot secondary to TCS (Figure 1A). We did not detect the occurrence of E130K/Pitx1 mutation in the 3 idiopathic CCF patients. Although Pitx1(+/-) mice share a descendant
with CCF-like mice [11], the mechanism of the downregulation of Pitx1 expression involved in clubfoot deformity is unclear.

Expression of Sirt1 decreases after the inhibition of Pitx1 in tendon cells.

This experiment used rat Achilles tendon cells to study the effect of the downregulation of Pitx1 expression on downstream pathways. After acquiring tendon cells from the Achilles tendon of newborn SD rats, we knocked down Pitx1 using Pitx1-siRNA-A in the tendon cells. Then, samples were collected. The downstream analysis of Pitx1 was based on quantitative proteomics using iTRAQ. Then, the identified differentially expressed proteins were analyzed by GO and KEGG analysis (Figure 1B, 1C). The proteomics results revealed that the Pitx1 knockdown efficiency was 75% (Figure 1D). Subsequently, the expression of Sirt1 was decreased by 39% in siRNA-treated tendon cells compared with nontreated tendon cells (Figure 1D). Despite the many changes in downstream signaling, we chose to focus on Sirt1. The results showed that the protein expression of Pitx1 and Sirt1 decreased after the inhibition of Pitx1 expression in tendon cells. Sirt1, Sirt6, and Sirt7 mainly have deacetylation functions in the nucleus [16]. Western blotting showed that the expression of Sirt1, but not of Sirt6 or Sirt7, was significantly downregulated after the inhibition of Pitx1 in tendon cells (Figure 2A). This suggests that the expression of Sirt1 is regulated by Pitx1.

Pitx1 binds to the promoter region of Sirt1, as evidenced by ChIP assays.

Pitx1 is a bicoid-related homeodomain transcription factor involved in vertebrate hindlimb development [17,18], but the relationship between Pitx1 and Sirt1 is unclear. We explored the possible mechanism by which Pitx1 regulates Sirt1 expression. Using a luciferase reporter assay, the Pitx1 gene was found to promote Sirt1 gene expression in tendon cells (Figure 2B). Furthermore, ChIP assays showed that Pitx1 binds to the promoter region of Sirt1 (Figure 2C). The Pitx1 binding motif is AGATTA [19]. There are 2 AGATTA sites in the rat Sirt1 promoter (Figure 2C). ChIP assays confirmed that Pitx1 binds to the Sirt1 promoter area, which contains the AGATTA motif. The downregulation of Sirt1 leads to activation of the RA pathway. An excess of retinoid compounds is associated with congenital malformations [20,21]. RA can induce CCF-like phenotypes in mice [12], and congenital malformations induced by RA are similar to those described in Sirt1 knockout mice [22,23]. However, the association of Sirt1 with CCF has not been reported.

Inhibition of Pitx1 activates the retinoic acid pathway through enhanced transport of RA

Retinoic acid (RA) is an essential signaling molecule that regulates multiple biological processes, including cell proliferation, differentiation, and death [24]. Previous studies have found that inhibition of Sirt1 leads to activation of the retinoic acid pathway [25], and we hypothesized that the inhibition of Pitx1 activates the retinoic acid pathway by downregulating the expression of Sirt1. Western blot analysis showed that, after the inhibition of Pitx1 expression in tendon cells, although there was no significant change in CRABP2, the expression of RARβ2, which is downstream of the retinoic acid pathway, increased significantly (Figure 3A). CRABP2 acts as a transport-binding protein that binds retinoic acid into the nucleus and regulates retinoic acid activity [26,27]. Immunofluorescence showed that the inhibition of Pitx1 increased the entry of CRABP2 into the nucleus (Figure 3B), suggesting an enhanced transport function. This may be related to the decreased deacetylation of CRABP2 by Sirt1, which results in enhanced activity, after the inhibition of Pitx1. This may be due to the downregulation in the deacetylation of CRABP2 by Sirt1 [25], which is downregulated after the inhibition of Pitx1, resulting in enhanced nuclear transport activity of CRABP2. After purification of the CRABP2 protein, the acetylation of CRABP2 was detected using anti-acetylated lysine and was found to be significantly increased (Figure 3C). The inhibition of Pitx1 led to increased acetylation of the CRABP2 protein, which enhanced the activity of CRABP2 by enhancing the transport of RA (Figure 3B, 3C). Therefore, after the suppression of Pitx1, the downregulation of Sirt1 reduced the deacetylation of CRABP2, resulting in the enhanced transport of RA and activation of the retinoic acid pathway.

Negative feedback between Pitx1/Sirt1 and the RA pathway

The treatment of tendon cells with low doses of retinoic acid leads to even greater enhanced expression of RARβ2 in the presence of the inhibition of Pitx1 (Figure 4A). We further examined the effect of RA on CRABP2 acetylation and RARβ2 expression after Pitx1 inhibition. We found that RA activated CRABP2, which acetylates and promotes the expression of RARβ2, after Pitx1 inhibition (Figure 4A). As the dose of retinoic acid applied to the tendon cells increased, Pitx1 protein expression decreased, RARβ2 expression increased, and CRABP2 acetylation increased (Figure 4B, 4C). Taken together, the above results show that the inhibition of Pitx1 activates the retinoic acid pathway by increasing the acetylation of CRABP2 and that retinoic acid-treated tendon cells exhibit downregulated Pitx1 expression, suggesting that positive feedback occurs between Pitx1/Sirt1 and RA/CRABP2 (Figure 4D).

Discussion

The Ponseti method for treating CCF has good clinical efficacy and leads to a favorable prognosis [28]. Other research groups have found some factors that influence the prognosis...
Figure 1. Expression of Pitx1 was detected in the human tibialis anterior tendon and its downstream effectors was detected by mass spectrometry. (A) Pitx1 expression was detected by immunohistochemistry in the tibialis anterior tendon of 3 idiopathic CCF patients and 2 neurogenic clubfoot patients (scale bar, 20 μm). After using siRNA to inhibit Pitx1 in tendon cells for 48 h, samples were collected. Downstream analysis of Pitx1 was based on quantitative proteomics using iTRAQ. Then, the differentially expressed proteins that were identified were analyzed by GO and KEGG analysis. (B) Differential protein function shown by GO analysis. (C) Differential protein function shown by a KEGG pathway analysis. (D) Partially differentially expressed proteins between Pitx1-knockdown rat tendon cells and normal tendon cells.
of CCF. The prognosis of CCF is related to the following factors: the patient’s age at initial treatment [29], the patient’s Pirani score at initial treatment [30,31], the number and type of casts required to obtain correction [32], whether percutaneous Achilles tenotomy (PAT) is required [33], and the effect of bracing and other factors [28]. Learning more about the pathogenesis of CCF is valuable for treating CCF and improving the prognosis. Although the cause of CCF is unknown, there is a mutation in the Pitx1 gene in familial CCF that leads to CCF in offspring [7]. Pitx1 not only affects the development of the lower limbs, but also the development of muscles and tendons in the lower limbs [10].

Pitx1(+/–) mice and RA-treated pregnant rats can produce offspring with a CCF-like phenotype [11,12], but the abnormal expression of Pitx1 in idiopathic CCF has not been reported. Further research on the downstream effects of Pitx1 is required.

The present results showed that, in a small case study, the expression of Pitx1 in the tibialis anterior tendon was lower in 3 patients with CCF than in control patients.

This may be an important factor in promoting the recurrence of CCF. Our mass spectrometry results showed that Sirt1 expression is downregulated after Pitx1 inhibition and that Sirt1 might be downstream of Pitx1 (Figure 1B–1D). Subsequent luciferase experiments confirmed that Pitx1 regulates Sirt1 expression (Figure 2B). ChIP experiments also confirmed that Pitx1 protein binds to the promoter region of Sirt1 (Figure 2C). Pitx1 binds to the promoter region of Sirt1 and promotes the transcription of Sirt1, which is a novel finding of downstream Pitx1 regulation.

Previous studies have confirmed that activation of the RA pathway is regulated by Sirt1 and that the nuclear entry of

Figure 2. Pitx1 regulates the expression of Sirt1. (A) After the inhibition of Pitx1 by 2 types of SiRNA in tendon cells, the expressions of Sirt1, Sirt6, and Sirt7 were detected by Western blotting. (B) The activity of the Sirt1 promoter region (region -2000/1) was analyzed by a luciferase assay in tendon cells after transfection with the Pitx1 gene. (C) ChIP showed that Pitx1 binds to the promoter region of Sirt1. The binding sequence is AGATTA.
Figure 3. After the inhibition of Pitx1 expression in tendon cells, the expression, distribution and acetylation of CRABP2 were detected. After the inhibition of Pitx1 by 2 types of siRNA in tendon cells, the cells were cultured for 48 h. (A) After 48 h of inhibition of Pitx1, the expressions of Sirt1, CRABP2, and RARβ2 were detected by Western blotting. (B) After 48 h of inhibition of Pitx1, cellular immunofluorescence was used to detect the expression and distribution of CRABP2. (C) After the inhibition of Pitx1 for 48 h followed by the purification of CRABP2 by using anti-CRABP2, the level of acetylation of CRABP2 was detected by anti-lysine by Western blot. Error bars indicate the mean±S.D. * p<0.05 was considered statistically significant.
Figure 4. There is an interaction between the RA pathway and Pitx1. (A) After the inhibition of Pitx1 by 2 types of siRNA in tendon cells, the cells were cultured for 48 h. Then, cells with inhibited Pitx1 expression and controls were treated with RA at a concentration of 20 nM. (B) After the treatment of tendon cells with different concentrations of RA for 48 h, Western blot analysis was used to detect Pitx1, CRABP2, and RARβ2 expression. (C) After the treatment of tendon cells with different concentrations of RA for 48 h followed by the purification of CRABP2 by using anti-CRABP2, the level of acetylation of CRABP2 was detected by anti-lysine by Western blot. (D) Schematic diagram of the mode of action between Pitx1/Sirt1 and the RA pathway. Error bars indicate the mean±S.D. * p<0.05 was considered statistically significant.
RA is inhibited by deacetylation of CRABP2 [25]. This experiment also found that Sirt1 expression is downregulated and CRABP2 acetylation and nuclear entry are increased after Pitx1 is inhibited (Figure 3A–3C).

Sirt1 knockout mice exhibit similar malformations to those induced by RA activation [22]. Although RA can cause CCF-like phenotypes in mice [12], such phenotypes have not been reported in Sirt1 knockout mice, which may be related to mice with CCF induced by single-gene knockout of Pitx1. This situation is similar to early research, in which foot deformities occurred but were ignored by the researchers. Further studies showed that RA can significantly inhibit the expression of Pitx1 and that Pitx1 inhibition promotes the nuclear translocation and downstream activation of RA, indicating that positive feedback occurs between RA and Pitx1.

As the dose of retinoic acid applied to the tendon cells increased, RARβ2 protein expression increased, which is consistent with previous research reports [34,35]. Previous research has shown that CRABP2, but not CRABP1, specifically binds with Sirt1 [25], so we studied CRABP2 translocation activity after Pitx1 was inhibited in tendon cells. As RARβ2 is downstream of the retinoic acid pathway, the expression of RARβ2 is detected to indicate the degree of activation of the retinoic acid pathway [36], and the inhibition of Pitx1 expression led to activation of the retinoic acid pathway through regulation of Sirt1 expression in tendon cells.

Our results suggest that the downregulation of Pitx1, which regulates development of the lower limbs, results in activation of the RA pathway in the lower limbs and is followed by foot dysplasia. It also suggests that the use of cosmetics containing RA or excessive use of vitamin A during pregnancy has the potential to cause deformities in offspring [24]. It needs to be further clarified whether there is a decrease in the expression of Pitx1 in the muscle and bone of CCF patients and if the decline is persistent; this could contribute to treatment strategies for CCF and improve the prognosis.

In summary, the downregulation of Pitx1 inhibits Sirt1 gene transcription and subsequent activation of the RA pathway in tendon cells. Decreasing the expression of Pitx1 is a possible mechanism for the occurrence of CCF. Further validation in clinical samples and animal models is required.

Conclusions

Pitx1 binds to the promoter region of Sirt1 and promotes the transcription of Sirt1. Inhibition of Pitx1 gene expression leads to downregulation of Sirt1 gene transcription and activation of the retinoic acid pathway in tendon cells. In addition, retinoic acid inhibits the expression of Pitx1 in tendon cells, suggesting that positive feedback occurs between the retinoic acid pathway and Pitx1, and this may be a possible mechanism by which low expression of Pitx1 leads to CCF.

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Conflict of interest

None.

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