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A novel screening model for the molecular drug for diabetes and obesity based on tyrosine phosphatase Shp2

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ABSTRACT

Tyrosine phosphatase Src-homology phosphotyrosyl phosphatase 2 (Shp2) was identified as a potential molecular target for therapeutic treatment of diabetes and obesity. However, there is still no systematic research on the enhancers for the Shp2 enzyme. The present study established a novel powerful model for the high-throughput screening of Shp2 enhancers and successfully identified a new specific Shp2 enhancer, oleanolic acid, from Chinese herbs.

Obesity and diabetes are very serious health problems that affect nearly 400 million people around the world. With continuous economic growth, these diseases are also increasing in frequency.[1] However, the development of therapies for obesity and diabetes is rather slow. Except for insulin, there have been very few effective treatments for diabetes.[2] Recently, molecular target therapy has shown great potential for disease therapy. Several medicines, such as metformin and thiazolidinediones that target AMPK and PPAR-γ, respectively, were used to treat diabetes and obesity.[2,3] However, these medicines are inadequate, so more molecular target medicines need be found.

Src-homology phosphotyrosyl phosphatase 2 (Shp2) is a cytoplasmic enzyme widely expressed in various tissues and cells.[4,5] As a tyrosine phosphatase, it regulates the tyrosine kinase receptor signal (EGF, insulin, and IGF-1) and also triggers several cytoplasmic kinase cascade pathways, such as RAS-RAF-MAPK, and PI3K/AKT, among others.[5,6] In the past 10 years, a disorder involving Shp2 activity was found to play a crucial role in abnormal development, tumorigenesis, and metabolic disease.[5,7,8] Several research on transgenic mice found that Shp2 activity is necessary to balance food intake and energy expenditure, and a blockage in Shp2 function leads to diabetes and obesity.[9–12] Deletions in the Shp2 protein in neuronal cells in the forehead of mice lead to obesity and diabetes with insulin and leptin resistance, hyperglycemia, hyperinsulinemia, hyperleptinemia, and other pathophysiological complications.[9,10] Mice with deletions in the Shp2 protein in muscle cells develop insulin resistance and dilated cardiomyopathy.[11,13] Selective deletions in the Shp2 protein in the pancreatic beta cells of mice exhibit defective glucose-stimulated insulin secretion and impaired glucose tolerance.[12] Thus, Shp2 is a potential target for the treatment of diabetes and obesity.[14] The substances that increase the activity of Shp2 are possible novel drugs for the prevention and treatment of metabolic diseases.

Shp2 is a protein tyrosine phosphatase (PTP) that catalyzes dephosphorylation at tyrosine residues.[5] PTP activity can be assessed through several assays based on different substrates, such as *p*-nitrophenyl phosphate (*p*NPP), phosphotyrosine, phosphotyrosyl peptides, and 8-diXuoro-4-methylumbelliferyl phosphate (DiFMUP).[15,16] Recently, some models have been developed to screen Shp2 inhibitors from chemical libraries based on PTP activity assays.[17–19] However, screening for Shp2 enhancers is still not reported.

Here, we established a novel model for the high-throughput screening of Shp2 enhancers using several optimality analyzes and successfully obtained a specific enhancer of Shp2 from a

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Chinese herbal extract library. This powerful screening system for Shp2 enhancers can help in the screening and identification of new chemical compounds involved in diabetes and obesity.

In this model, a purified protein of full-length Shp2 (see 'PTP protein purification' in Supplementary data 1) was selected rather than the protein of the PTP domain of Shp2 (PTP-Shp2), which was usually employed for the screening of Shp2 inhibitors.[20] Shp2 enzyme activity is self-regulated by the interaction between its N-terminal SH2 domain and the PTP domain in its natural structure.[5] When growth factors or cytokines stimulate cells, tyrosine residues attract SH2 domains and free the PTP domain, which has phosphatase activity.[5] Thus, in our assay model, the basal activity of full-length Shp2 protein is low, and activity enhancement was easy to check when the extracts/compounds stop or reduce the self-repression of Shp2.

PTP activity can be assayed using several substrates, such as *p*-nitrophenyl phosphate (*p*NPP), phosphotyrosine, phosphotyrosyl peptides, 4-methyl-7-hydroxycoumarinyl phosphate (MUP), and 3,6-Xuorescein diphosphate (FDP).[15,16] However, most substrates are not sensitive enough to detect basal Shp2 activity. Therefore, 8-diXuoro-4-methylumbelliferyl phosphate (DiFMUP), a fluorogenic substrate, that is, very sensitive to PTP enzymes, was selected.[21] Then, we optimized the experiment condition and determined the optimal concentration of DiFMUP (20 μM) and the Shp2 enzyme (42 μM) sufficient for the full activity of the latter. DiFMUP is sensitive to temperature, so all experiments were kept at 20–25 °C.[21]

Based on this optimal PTP assay model, we selected the natural products of Chinese herbs rather than chemical compounds to screen for Shp2 enhancers following the process described in Figure 1A. Chinese herbal medicine is a vast drug resource that contains a large number of natural therapeutic substances. However, the composition of Chinese herbs is too complex. The isolation of natural products from Chinese herbs requires a lot of work. Hence, a complete library of natural products from Chinese herbs is still unavailable. Thus, we here firstly selected an extract library of Chinese herbs (see 'Chinese herbal extract library' in Supplementary data 1) to screened for positive extracts. Then, we selected the Chinese herb that contains the positive extracts and isolated its positive compounds sequentially (details was described in 'Extraction and isolation' in Supplementary data 1). In this model, a positive extract or compound is one that enhanced Shp2 activity by more than 30%. In each cyclical step, the mixture was first separated into several extracts using two chemical solvents with different ratios (Fig. 2), and then all extracts were evaluated for their Shp2-enhancing effect (data are shown in Fig. 3). The positive extracts were selected to enter into the next step.

Ten micromolar doses of each extract () in the library were used. Of 2356 total extracts, 2266 (96.1%) did not enhance Shp2 activity (<5% enhancement), whereas only 75 (3.2%) enhanced Shp2 by 5–30%. Only 15 extracts (0.7%) enhanced Shp2 activity

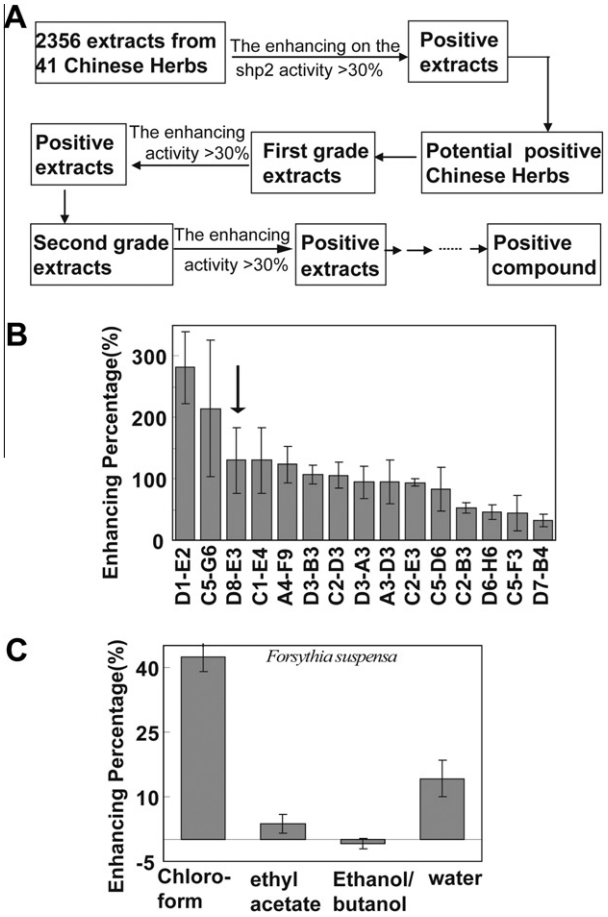

Figure 1. Screening and identification of Chinese herbs that promote Shp2 catalytic activity. (A) Overview of the process for screening for enhancer compounds of Shp2 activity from Chinese Herbs. (B) Fifteen positive extracts were selected from 2356 extracts in the library of Chinese herbs. D8-E3 is an extract from *F. suspensa*. (C) The component substances of *F. suspensa* were first extracted using chloroform, ethyl acetate, butanol, and water. Only chloroform extracts showed positive enhancement (42% increase) of Shp2 activity. Here, the concentration of each extract is 10 μM (the molecular weight was supposed as 1000). Each experiment was carried out in triplicate, and the enhancement percentages are expressed as the mean ± standard deviation from three experiments.

by more than 30% (Fig. 1B). D8-E3 is a chloroform extract from *Forsythia suspensa*, a famous Chinese herb used to treat several diseases including diabetes. Hence, *F. suspensa* was selected for the isolation and identification of Shp2-enhancing natural products. The component substances of *F. suspensa* were first extracted using chloroform, ethyl acetate, butanol, and water, and then the activity of each extract (10 μM, the molecular weight was supposed as 1000) was assayed. Only the chloroform extracts enhanced (42%) Shp2 activity (Fig. 1C).

Then, the effects of the chloroform extracts from *F. suspensa* on blood sugar level was evaluated with an STZ-induced diabetes mice model (see 'Diabetes mice model and the treatment of extracts' in Supplementary data 1). This model can be employed to assess T1DM mechanisms, to screen for potential therapies for diabetes, and to evaluate therapeutic options.[22] The results are shown in Table 1. In diabetic mice, the average blood glucose level was around 19.29 mM at day 0, 25.02 mM at day 7, and 31.28 mM at day 14. The chloroform extracts of *F. suspensa* clearly suppressed the increase in blood glucose. In mice with low doses of the extract, the blood glucose at days 7 and 14 was 23.61 and 25.81 mM, respectively, which is lower than that of diabetic mice (p <0.05) (Table 1). Medium doses also decreased the blood glucose in mice (24.82 ∝ 25.02, 26.78 ∝ 31.28) (p <0.05) (Table 1). Interestingly, a

Table 1
The chloroform extract from *Forsythia suspensa* (FSC) decreased the blood glucose in diabetes model mice

Group	Blood glucose (mM)		
	0 day	7 day	14 day
Normal	8.99 ± 1.04	7.04 ± 1.44	7.66 ± 1.18
Diabetes	19.29 ± 6.31	25.02 ± 2.61 *	31.28 ± 2.59 **
100 mg/kg FSC	19.61 ± 5.71	23.61 ± 3.75[a] *	25.81 ± 5.68 *
400 mg/kg FSC	20.96 ± 3.43	24.82 ± 5.18 *	26.78 ± 7.43 **
800 mg/kg FSC	19.37 ± 4.27	22.37 ± 1.93 **	20.5 ± 2.95 *
Metformin (111.5 mg/kg)	17.41 ± 2.78	18.95 ± 4.17	28.86 ± 3.03

[a] The p value in T test by compare with the blood glucose in diabetes group.$^{*}p$ <0.05,$^{**}p$ <0.01.

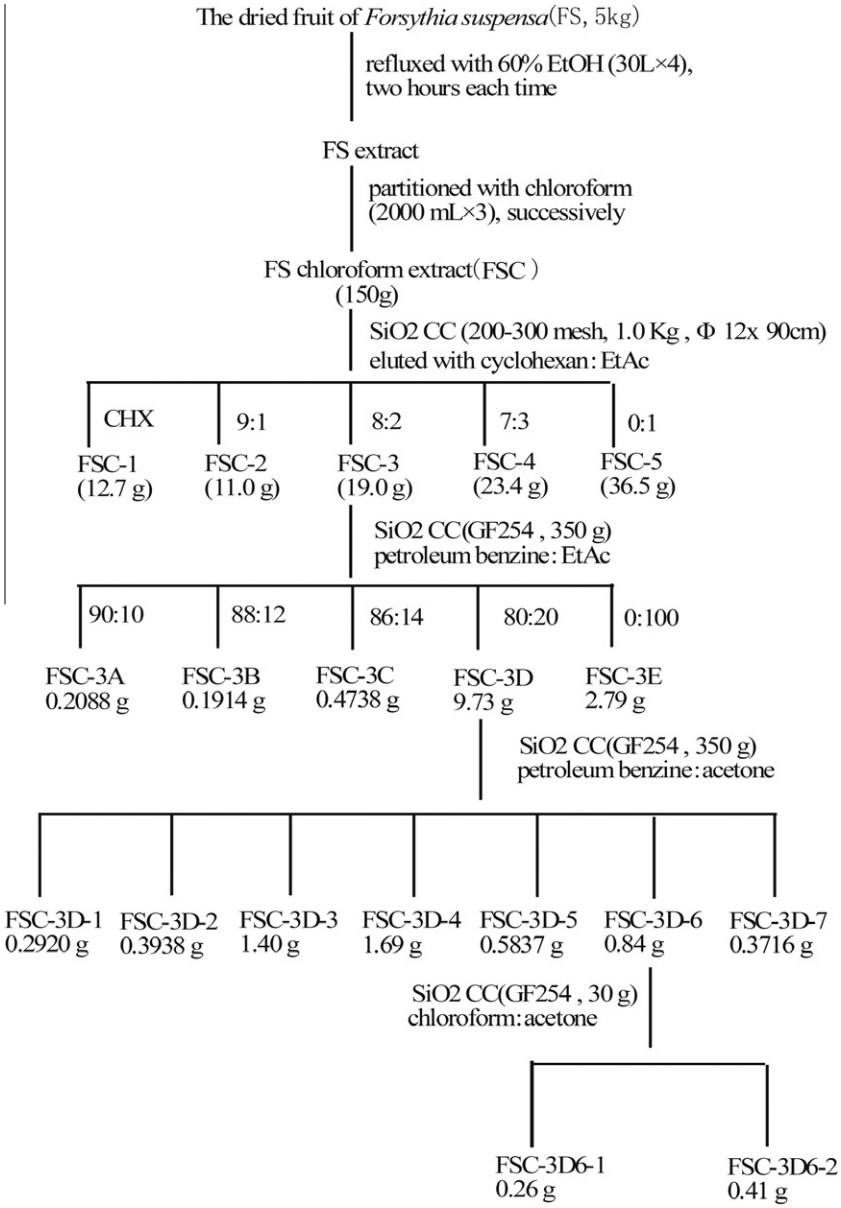

Figure 2. Procedure for extracting the components of *F. suspensa* using chemical solvents. The positive compound was isolated through several cycles. In each cycle, the mixture was first separated into several extracts using different ratios of two chemical solvents, and then all extracts were evaluated for their enhancing effect on Shp2 activity (data are shown in Fig. 3). The positive extracts were selected to enter into the next cycle.

high dose of extracts shows remarkably reduced effects on blood glucose (p <0.01) (Table 1). Especially at day 14, the blood glucose level dropped to 20.5 mM, nearly similar to that at day 0.

The results showed that the chloroform extracts of *F. suspensa* blocked the abnormal increase in blood glucose level in diabetes mice and suggested that *F. suspensa* has a bio-effect of reducing blood glucose. Based on its effect on Shp2 activity, *F suspensa* may regulate the blood glucose level by enhancing Shp2 bio-effects, such as the promotion of insulin secretion, activation of leptin signals, and so on.[10,12,14] Although *F. suspensa* is a traditional Chinese herb for the treatment of diabetes, its mechanism of action is still unknown. Our results, therefore, provide some insight into its mechanism of action.

To obtain the positive nature product compound of Shp2, 150 g of chloroform extracts from *F. suspensa* was subjected to extract next-grade fractions (Fig. 2) (details was described in 'Extraction and isolation' in Supplementary data 1). In these experiments, the concentration of each extract is 10 μM (the molecular weight was supposed as 1000). Five fractions (FSC1, FSC2, FSC3, FSC4, and FSC5) were eluted from FSC. Their effects on Shp2 enzyme activity were then assessed using Shp2 phosphatase assay system. The results show that FSC1, FSC2, FSC3, and FSC5 enhanced Shp2 activity by more than 30% (Fig. 3A). Considering the constituents' complexity of these fractions, FSC3 (19.0 g) was first selected to enter the next step. Other positive extracts were assayed later. From FSC3, five extracts (FSC-3A–FSC-E) were obtained (Fig. 2); FSC-3D and FSC-3E tested positive for Shp2 activity (Fig. 3B). FSC-3D was the main component of FSC3, so FSC-3D was chosen for the next experiments. Eight fractions (FSC-3D-1–FSC-3D-8) were purified (Fig. 2) from FSC-3D, and four of them (FSC-3D-3–FSC-3D-6) increased Shp2 activity by more than 30% (Fig. 3C). FSC-3D-6 contained the two compounds FSC-3D-6-1 and FSC-3D-6-2 (Fig. 2). FSC-3D-6-1 showed very strong Shp2 enzyme activity (85.1%), whereas FSC-3D-6-2 activity was low (18.9%) (Fig. 3D).

To check whether the enhancing effects of FSC-3D-6-1 on Shp2 activity were specific, three other PTP proteins, Shp1, Vhr, and

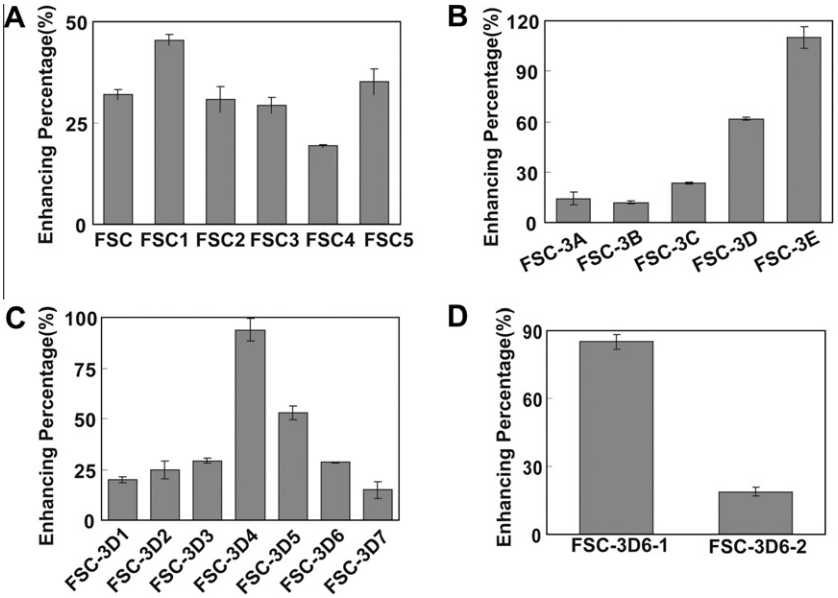

Figure 3. Enhancing effect of the extracts from *F. suspensa* on Shp2 enzyme activity. (A) First-grade extracts from the chloroform extracts of *F. suspensa* (FSC). (B) Second-grade extracts from FSC. (C) Third-grade extracts from FSC. (D) Fourth-grade extracts (compounds from FSC). The concentration of each extract is 10 μM (the molecular weight was supposed as 1000). All experiments were performed in triplicate, and the enhancement percentages are expressed as the mean ± standard deviation from three experiments.

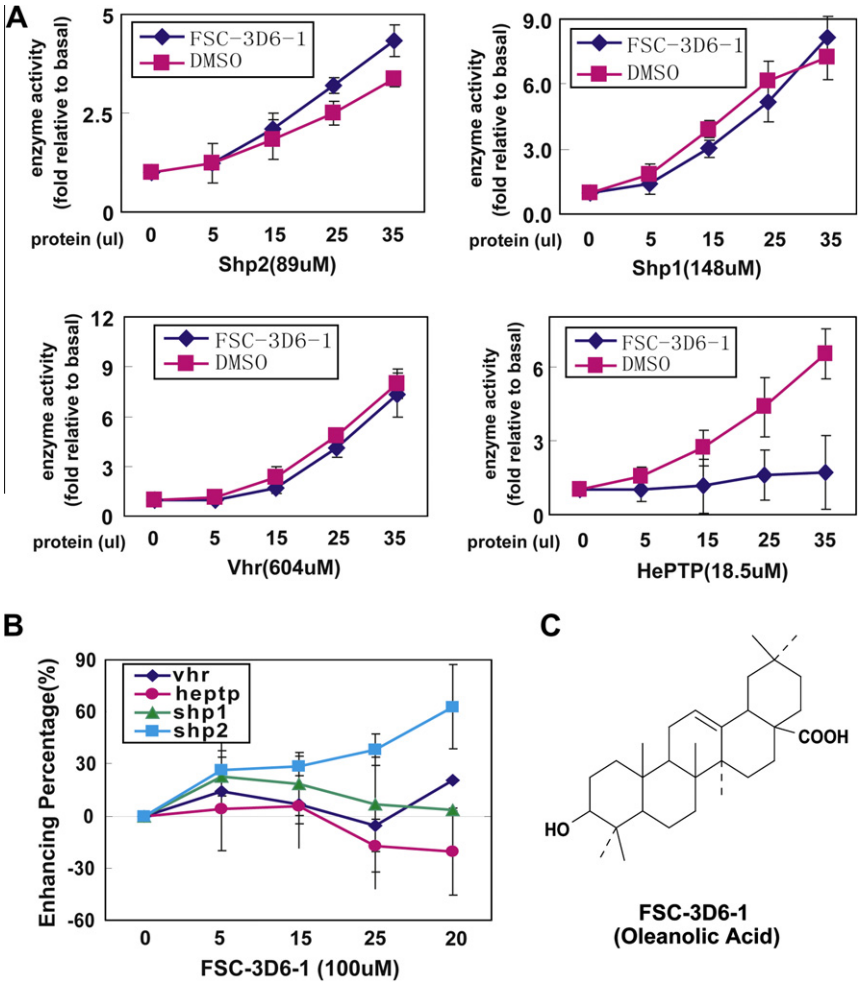

Figure 4. Identification of a specific enhancer for Shp2 activity. (A) The single compound FSC-3D6-1 (10 μM) specifically increased the enzyme activity of the Shp2 protein without enhancing the activity of the other PTP enzymes (shp1, Vhr, and HePTP). The *Y*-axis is the average enzyme activity relative to basal enzyme activity from three experiments. (B) FSC-3D6-1 specifically promoted Shp2 activity in a dose-dependent manner, and increasing concentrations of FSC-3D6-1 did not enhance other PTP enzymes. The enhancement percentages are expressed as the mean ± standard deviation from three experiments. (C) FSC-3D6-1 was identified as oleanolic acid (OA).

HePTP, were used.[23] Using the same substrates (10 μM), the effects of FSC-3D-6-1 on the activity of different doses of PTP proteins (Shp2, shp1, HePTP, and Vhr) were first assayed. Basic Shp2 activity was low, so its enzyme increases slowly with an increase in Shp2 protein concentration. When Shp2 activity was enhanced by the extracts, the increase in activity was rapid. In the results shown in Figure 4A, FSC-3D-6-1 remarkably increased Shp2 activity without affecting other PTP enzymes.

Then the effects of different concentrations of FSC-3D-6-1 on the PTP enzyme were also tested, revealing that FSC-3D-6-1 increased Shp2 activity at a dose-dependent manner (Fig. 4B, square curve). Meanwhile, FSC-3D-6-1 did not enhance Shp1, Vhr, and HePTP at other concentrations.

The compound structure was analyzed using the following parameters: melting point, NMR spectra, and ESIMS spectra (see 'Identification of the chemical structure' in Supplementary data 1). Then all parameters of the compounds were compared with reference information (Supplementary data 2).[24] The results show that compound L3-D6-1 had similar spectral data to OA, suggesting that they contained the same chemical structure (Fig. 4C).

OA is a pentacyclic triterpene present in many plants, including some Chinese herbs, and is used in a wide range of treatments for inflammation, hyperlipidemia, and hypoglycemia.[25–27] OA is also known to mediate glucose metabolism. The blood sugar of hyperglycemic rats can be decreased by OA.[25] OA treatment of HFD-fed mice results in significant decreases in body weight, abdominal fat, and blood glucose and lipids.[28,29] Furthermore, OA improves obesity-associated insulin resistance and hyperlipidemia.[17] Recently, OA was used to treat STZ-induced diabetes in mice.[7,30] These results demonstrate that OA is a multifunctional glucose metabolic regulator and also suggest that our high-throughout screening system is capable of screening for Shp2 enhancer.

The mechanism of OA is complex and multi-targeted,[17] such as blocking of intestinal glucose absorption by inhibiting alpha-glucosidase activity,[31] amelioration of the insulin pathway by suppressing the inhibitory activity of protein tyrosine phosphatase 1B (PTP 1B),[32] and repression of 11β-hydroxysteroid dehydrogenase type 1 (11beta-HSD1) in the liver and peripheral tissues.[33] Our results identified OA as an enhancer for Shp2 enzyme activity which mediates glucose metabolism and provides a novel mechanism for OA bio-effects.

Several studies using transgenetic mice models demonstrated that reduced or absent Shp2 activity causes disorders in glucose and fat metabolism, which develop into diabetes and obesity. Hence, the substances that enhance Shp2 activity are regarded as potential medicine for the prevention and treatment of diabetes by restoring normal Shp2 activity. Our experiment is the first to use a screening system for Shp2 enhancers and to identify successfully a novel specific enhancer for Shp2 activity from *F. suspensa*. This powerful system is expected to help identify more compounds for the treatment of metabolic diseases.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.11.049.

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