Puerarin Suppresses the Self-Renewal of Murine Embryonic Stem Cells by Inhibition of REST-MiR-21 Regulatory Pathway

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Key Words
Puerarin • Murine embryonic stem cells • Self-renewal • REST • MiR-21

Abstract
\textbf{Background/Aims:} Puerarin shows a wide range of biological activities, including affecting the cardiac differentiation from murine embryonic stem (mES) cells. However, little is known about its effect and mechanism of action on the self-renewal of mES cells. This study aimed to determine the effect of puerarin on the self-renewal and pluripotency of mES cells and its underlying mechanisms. \textbf{Methods:} RT-PCR and real-time PCR were used to detect the transcripts of core transcription factors, specific markers for multiple lineages, REST and microRNA-21 (miR-21). Colony-forming assay was performed to estimate the self-renewal capacity of mES cells. Western blotting and wortmannin were employed to explore the role of PI3K/Akt signaling pathway in the inhibitory action of puerarin on REST transcript. Transfected mES cells with antagomir21 were used to confirm the role of miR-21 in the action of puerarin on cell self-renewal. \textbf{Results:} Puerarin significantly decreased the percentage of the self-renewal colonies, and suppressed the transcripts of Oct4, Nanog, Sox2, c-Myc and REST. Besides, PECAM, NCAM and miR-21 were up-regulated both under the self-renewal conditions and at day 4 of differentiation. The PI3K inhibitor wortmannin successfully reversed the mRNA expression changes of REST, Nanog and Sox2. Transfection of antagomir21 efficiently reversed the effects of puerarin on mES cells self-renewal. \textbf{Conclusion:} Inhibition of REST-miR-21 regulatory pathway may be the key mechanism of puerarin-induced suppression of mES cells self-renewal.

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Introduction

Embryonic stem (ES) cells have been widely considered as one of the promising cell sources which are not only used for cell replacement therapy, but also used as the best platform for the developmental research, drug discovery and screening [1-3]. ES cells are characterized by the potential for both self-renewal and differentiation into all three germ layers of the body [4]. The self-renewal is one of the interests in ES cell research, which is closely related to the expression of master transcription factors Oct4, Sox2, Nanog, c-Myc [5]. In murine ES (mES) cells, the expression of these master transcription factors is mainly regulated by the crosstalk between the JAK/STAT3, PI3K/Akt and Ras/ERK signaling pathways [5-7].

The neuronal repressor RE1-silencing transcription factor (REST) is a major transcriptional repressor of neurogenesis, and participates in the neural stem cells self-renewal [8]. It is a protein containing nine Cys2/His2 zinc finger, having a DNA binding domain and a lysine-rich region, a proline-rich region and two transcriptional repression domains (N terminal and the C-terminal repression domain)[9]. The gene of REST locates at the 5th chromosome of mES cells, and has 5 exons [10]. REST binds to the RE1 element, which is a 21-23 base pair sequence in its target genes promoters [11]. REST is highly expressed in mES cells, however, its role in maintaining self-renewal of mES cells is not fully clear due to conflicting data reported by different groups, and extended effort is still needed to fully understand the underlying mechanism [12-15] . Of note, REST forms part of the Nonog-Oct4 complex [16], and has been predicted to be a major component of Oct4-Sox2-Nanog network in mES cells [17]. Knocking down exon1 or 2 of REST in mES cells (YHC334 and RRC160 cells) results in a reduction of REST and specifically up-regulation of its downstream target microRNA-21 (miR-21), subsequently leads to the loss of Oct4, Nanog, Sox2 and c-Myc, and a reciprocal gain of specific markers for multiple lineages [15]. Consistently, in ES cells and the whole adult brain of Down’s syndrome mice, the decrease in REST levels promotes down-regulation of Oct4, Nanog and Sox2 [18]. By using a mathematical model, a recent effort provides new evidence to further confirm the significant role of the REST-miR-21 regulatory pathway in self-renewal and differentiation of ES cells [19]. Taken together, this evidence indicates that REST is an important factor of the interconnected regulatory network that maintains the self-renewal in mES cells [15].

Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-(β-D-Glucopyranoside)] is a natural phytoestrogen found in the herb radix pueraria or pueraria lobata (Willd.), which has been applied in traditional Chinese medicine for thousands of years[20]. In general, 400mg puerarin in 250 ml of 5% Glucose injection (the maximum plasma concentration of puerarin is about 1-3 mM) are injected intravenously once a day in the treatment of the cardiovascular diseases. Nevertheless, further in vitro studies are needed to answer the underlying mechanism of the therapeutically effects of puerarin. Up to date, puerarin research is mainly focused on its action on the proliferation and differentiation of various types of cells, and the working dose of puerarin is about 50-200 µM, which is significantly lower than that applied in clinic. Puerarin increases the proliferation of human osteoblastic MG-63 cells [21], but reduces the proliferation of high-glucose-and diabetes-induced vascular smooth muscle cell [22]. Puerarin promotes the in vitro osteoblasts differentiation in osteoblast-like UMR106 cells [23], and induces adipocyte differentiation of 3T3-L1 preadipocytes [24]. Our previous data have revealed that puerarin promotes in vitro cardiac differentiation, enhances the specialization of mES cells into ventricular-like cardiomyocytes [2], and facilitates the development of t-tubules in mES cell-derived cardiomyocytes [25]. However, little is known whether puerarin could exert effects on mES cells self-renewal.

In the present study, we aimed to determine the effect of puerarin on the self-renewal and pluripotency of mES cells and its underlying mechanisms. Our findings suggest that puerarin inhibits the self-renewal of mES cells via REST-miR-21 regulatory pathway, revealing new biological effect and underlying mechanism of puerarin, and providing new clue for the future application of puerarin.
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Materials and Methods

Cells culture and differentiation of mES cells

The mES cell line D3 (GRL-1934, ATCC, USA) was cultured on mitomycin C inactivated mouse embryonic fibroblasts under self-renewal conditions, which composed of Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 1000 IU/mL leukemia inhibitory factor (ESGRO, Chemicon, USA), 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, 0.1 mM β-mercaptoethanol (β-Me), and 1% non-essential amino acids (NEAA) as previously described[1]. Cells were passaged every 48h. The differentiation of mES cells was initiated by the generation of embryonic bodies (EBs) using typical hanging drop method in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS, 2 mM L-glutamine, 100 units/mL penicillin-streptomycin, 0.1 mM β-Me, and 1% NEAA. Puerarin (National Institutes For Food and Drug Control, China) or 0.05% dimethyl sulfoxide (DMSO, Sigma, USA) were applied under self-renewal conditions or during differentiation. All cultivation medium and reagents were purchased from Gibco if not otherwise indicated.

Calculation of the population doubling times

Cells were seeded in a fixed number of cells (2×10^4 cells/well) on gelatin-coated 6-well-plates, and grew for 48h. Cells were trypsinized and counted using trypan blue and a haemocytometer. The population doubling times were calculated as described previously [26].

Colony-forming assay

500 mES cells were seeded per well of a 6-well plate under self-renewal conditions. After 7 days cultivation, the colonies were stained with alkaline phosphate dye as previously described [27]. The percentage of the self-renewal colonies was calculated. Of note, during the whole procedure culture medium was needed to be changed every three days.

RT-PCR and Quantitative real-time PCR

Total mRNA was extracted from mES cells and 4-days-old EBs with Trizol (Invitrogen) following the instructions of the manufacturer, and RT-PCR was performed using PCR Master Mix. The PCR products were separated by agarose gel electrophoresis and analysed using Image J software.

Real-time PCR was carried out in the Mx3000P real-time PCR system (Stratagene, USA) with SYBR Green Real time PCR Master Mix plus (Toyobo, Japan). Bulge-LoopTM microRNAs (miRs) primer for U6

Table 1. Primers for RT-PCR and Real-time PCR analysis

| Genes    | Primer sequence(sence/antisence) | Annealing temp (°C) | Cycles | Products size(bp) |
|----------|----------------------------------|---------------------|--------|------------------|
| GAPDH    | AAC TTT GGC ATT GTG GAA GG       | 54                  | 30     | 132              |
| Nanog    | GGA TGC AGG GAT GTT CT           | 58                  | 30     | 128              |
| Oct4     | CAGCGCAAGACACATCTTCGTG           | 58                  | 30     | 138              |
| Sox2     | AACCCCAAGATGCCAAACTCT            | 53                  | 30     | 203              |
| c-Myc    | GCTTCTCCATCCTATGTGGG             | 53                  | 30     | 116              |
| REST     | AGGAGGTACCACCTGGGAGAA            | 54                  | 28     | 116              |
| PECAM    | CTGAAAGATGTCCGAAGTA             | 54                  | 33     | 115              |
| SOX17    | GGACCGCTACGTGGG               | 52                  | 33     | 128              |
| brachyury | GCCAAAGGCTACAACCTGAAGG        | 58                  | 33     | 128              |
| NCAM     | ACCACGTCACCAACTCCT             | 56                  | 33     | 102              |
and miR-21 (RIBOBIO, China) were used according to the manufacturer’s protocols. The transcript levels of GAPDH or U6 were used for internal normalization. Relative quantification of PCR products was calculated using the 2^{ΔΔCT} method [28] and normalized by control. The primers used for RT-PCR and Real-time PCR were listed in Table 1.

**Western Blotting**

To investigate the effect of puerarin on the phosphorylation of Akt and ERK1/2, the key protein in PI3K/Akt and MEK/ERK signaling pathways, mES cells were pretreated in Opti-MEM for 2 hours, and then cultured under self-renewal conditions with or without puerarin treatment. Protein was extracted from mES cells at different timepoint (0 and 10 minutes, 0.5, 1 and 2 hours, respectively). Western blots were performed using 50 µg protein Membranes were blocked in 5% non-fat milk PBS-T (0.05% Tween-20), and incubated with antibodies to specific protein overnight followed by incubation with the secondary antibody for 1 h. Antiphospho-Ser^{473} Akt MAb was obtained from Abcam (1:5000, http://www.abcam.cn), antiphospho-ERK-1/2 MAb was obtained from Cell Signaling Technology (1:2000, Beverly, MA, http://www.cellsignal.com).

**Cell Signaling Inhibitors**

Specific inhibitors were used to probe the possible cell signaling pathway mediating the effect of puerarin on REST transcript level. MES cells were pretreated in Opti-MEM (Invitrogen, USA) for 2 hours, and then cultured under self-renewal conditions in the presence of the PI3K inhibitor wortmannin 1 µM (Sigma-Aldrich, USA). The puerarin was added after 2 hours treatment with inhibitors. Total mRNA was extracted from mES cells after puerarin treatment for 48 hours, and the transcript level of REST, Nanog and Sox2 were assessed by real-time PCR.

**Cell Transfection**

200,000 mES cells were seeded on 35mm culture dish overnight under the self-renewal conditions, and transfected with Opti-MEM containing 100 nM antagomir21 (RIBOBIO, China) or antagomir negative control, respectively for 5 hours. Then cells were further cultured under self-renewal conditions with or without puerarin for 48 hours, and were ready to be used for real-time PCR analysis and alkaline phosphatase staining.

**Statistical analysis**

All data were expressed as mean ± SEM for at least three independent experiments. The significance of difference between the means was evaluated by student’s unpaired t-test or ANOVA. And the values p < 0.05 was considered to be statistically significant.

**Results**

**Puerarin inhibits the self-renewal of mES cells**

Under the self-renewal conditions, moderate expression of core stemness genes can maintain self-renewal and pluripotency of mES cells. And Oct4 as a basic transcription factor is indispensable for maintaining the essential characteristics of mES cells. Firstly, we studied whether puerarin treatment affects Oct4 transcript level of mES cells under the self-renewal conditions. Puerarin down-regulated the expression level of Oct4 in a dose-dependent manner (IC50 was 44.67 ± 3.54 µM, Fig. 1A) without affecting the population doubling times of undifferentiated mES cells (Table 2). 100 µM, the dose of maximum effect of puerarin, was applied in the following experiments. Puerarin also significantly down-regulated the transcript levels of other core transcription factor Nanog, Sox2 and c-Myc by approximately 60% ~ 70% (p<0.05, n = 5) (Fig. 1B). The colony-forming assay was performed to further estimate the self-renewal capacity of mES cells. As shown in Fig. 1C, the self-renewing colonies showed a distinct and compact structure, and strong alkaline phosphatase activity, while the differentiated colonies had a spread-out morphology and less alkaline phosphatase activity. Under the self-renewal conditions, the percentage of self-
renewal colonies in puerarin group (59 ± 3%) was lower than that in the control group (87 ± 2%, p<0.05, n = 7) (Fig. 1D). These data suggest that puerarin-treated mES cells may be differentiating and losing their self-renewal capacity.

Puerarin promotes the differentiation of mES cells toward endoderm and ectoderm.

We next studied the effects of puerarin on the pluripotency of mES cells. We performed RT-PCR and real-time PCR to detect the expression of endoderm differentiation marker Sox17 and PECAM, mesendoderm differentiation marker T-brachyury and ectoderm differentiation marker NCAM under both self-renewal conditions and differentiation conditions. We found

### Table 2. The effect of puerarin on the population doubling times of undifferentiated mES cells

|                      | control          | The dose of puerarin (µM) |
|----------------------|------------------|--------------------------|
|                      |                  | 1           | 10         | 100        | 1000        |
| Starting cell numbers (10⁵) | 0.2              | 0.2         | 0.2        | 0.2         | 0.2         |
| Harvesting cell numbers (10⁵) | 1.95±0.45       | 2.10±0.41  | 2.14±0.55  | 2.47±0.46  | 2.17±0.40  |
| Population doubling time (h) | 15.58±2.19     | 14.74±1.59 | 15.26±2.46 | 13.67±1.27 | 14.41±1.36 |
that puerarin-treated mES cell but not control cells expressed all the above mentioned markers under the self-renewal condition (Fig. 1E). Moreover, the transcript levels of PECAM and NCAM were up-regulated compared with control group (*, p<0.05, n = 3) at day 4 of differentiation (Fig. 1F). These data demonstrate that puerarin decreases the pluripotency of mES cells, and promotes the differentiation of mES cells toward endoderm and ectoderm.

**Puerarin suppresses REST transcription of mES cells under the self-renewal conditions via activation of the PI3K/Akt signaling pathways**

We next studied whether REST/miR-21 regulation mediates the puerarin-induced loss of self-renewal. We found that puerarin significantly down-regulated the transcript level of REST in dose-dependent manner under self-renewal condition. This inhibitory effect was also observed under differentiation conditions (p<0.05, n = 3, Fig. 2B). To understand how puerarin suppresses REST transcription, we investigated the PI3K/Akt or/and MEK/ERK signaling pathways induced by puerarin. Under self-renewal condition, puerarin treatment caused a gradual increase in Scr473 Akt phosphorylation within 2 hours, but did not affect the phosphorylation of ERK except 2 hours after puerarin treatment (Fig. 2C). Wortmannin (W, 1 µM), a PI3K specific inhibitor, successfully rescued the transcript levels of REST, Nanog and Sox2 under the self-renewal condition (Fig. 2D). These results demonstrate that the PI3K/Akt signaling pathway involves in the puerarin-induced REST transcript suppression and subsequently decreased self-renewal of mES cells under the self-renewal conditions.
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Puerarin up-regulates miR-21 of mES cells, and antagonmir21 reverses the effects of puerarin on mES cells

MiR-21 is one of downstream target of REST. We found that puerarin significantly up-regulated miR-21 in mES cells under both self-renewal and differentiation conditions. The expression level of miR-21 in mES cells under both self-renewal and differentiation conditions. The concentration of antagonmir21 and negative control is 100 nM. (C) Representative images of alkaline phosphatase staining of mES colonies treated by puerarin with or without antagonmir21. (D) Effect of antagonmir-21 on the percentages of self-renewing colonies of puerarin treated mES cells. Scale bar = 400 µm. (n = 7). All data are presented as mean ± SEM. *, p<0.05 vs. control group.

Discussion

The key findings of the present study were: (1) puerarin significantly decreased the percentage of the self-renewal colonies, and suppressed the transcripts of Oct4, Nanog, Sox2, c-Myc and REST; (2) PECAM, NCAM and miR-21 were significantly up-regulated both under the self-renewal conditions and at day 4 of differentiation; (3) the PI3K inhibitor wortmannin successfully reversed the mRNA expression changes of REST, Nanog and Sox2;
(4) transfection of antagomir21 efficiently reversed the effects of puerarin on mES cells self-renewal.

The majority of biological effects of puerarin on different cells had been proven to be related to the regulation of PI3K/Akt or/and ERK signaling pathways [21, 29]. Puerarin promotes the proliferation of endothelial progenitor cells and bone formation from rat calvaria osteoblasts by activation of PI3K/Akt signaling pathway [30, 31], stimulates proliferation and differentiation in human osteoblastic MG-63 cells via ERK and PI3K/Akt activation [21], and exerts cytoprotection action via estrogen receptor-dependent upregulation of PI3K/Akt and H0-1 [29]. Here, we found that puerarin activates PI3K/Akt signaling in mES cells under the self-renewal conditions. Previous evidence demonstrates that the activation of PI3K/Akt signaling is important to maintain the self-renewal mES cells in the presence of LIF and feeder cells [32, 33]. The activation of PI3K/Akt signaling maintains Oct4 expression and self-renewal of mES cells via activation of Klf4 and Tbx3 [5]. Application of specific inhibitor of PI3K/Akt signaling during cultivation of different mES cells lines (E14tg2a, CCE and IOUD2) inhibits their self-renewal [32, 33]. Obviously, these above mentioned evidence cannot explain why puerarin activates PI3K/Akt signaling but suppresses the self-renewal of mES cells. Alternatively, we hypothesized that another regulatory pathway might involve in the puerarin-induced activation of PI3K/Akt signaling and suppression of mES self-renewal.

Despite of controversies surrounding the role of REST in maintaining the self-renewal in mES cells [12, 14-16, 19], REST has been suggested to modulate the balance of self-renewal and differentiation of both ES cells and neural stem cells [8, 19, 34]. By establishing mathematical model, a recent report confirms that the changes of REST expression level negatively affect the expression of miR21, substantially results in the bistable switching curve shift and accordingly gives more opportunities of ES cells in the state of self-renewal or differentiation [19]. Consistently, in the present study, we found that puerarin treatment inhibits the REST-miR-21 regulatory pathway, and correspondingly suppresses the self-renewal and promotes the differentiation of mES cells toward endoderm and ectoderm lineages. The PI3K specific inhibitor wortmannin successfully reverses the mRNA expression changes of REST, Nanog and Sox2. Nevertheless, the level of REST in various types of cells has been found to depend on two mechanisms: gene transcription controlled by Wnt signaling and turnover of the protein, the latter being essential especially for the decrease of REST taking place during neuronal precursor maturation. Here, we only studied and observed the transcriptional change of REST controlled by the PI3K/Akt cascade; however, the step directly involved is needed to be further identified. The detail downstream signal pathway by which puerarin affects REST transcription and the downstream targets of miR21 are still unknown. Additionally, there is crosstalk between the PI3K/Akt, ERK, and Wnt/β-catenin signaling pathway participating in maintenance of mES cells self-renewal [33, 35]. Furthermore, using genetic complementation and knockdown approaches, Tomasoni R et al. found that a signaling loop of REST, GTase-activating protein tuberin (TSC2) and the transcriptional co-factor β-catenin determines proliferation and function of PC12 neural cells [36]. Whether purarin-induced activation of PI3K signaling affects TSC2 and Wnt/β-catenin signaling pathway is still unknown. Besides, further studies on human pluripotent stem cells are needed to explore and confirm our findings because of substantial differences between animal and humans.

In conclusion, our data suggest that puerarin inhibits the self-renewal and promotes the differentiation of mES cells toward endoderm and ectoderm lineages via inhibition of the REST-miR-21 regulatory pathway. Our findings provide new understanding of the biological effects of puerarin on the self-renewal of pluripotent stem cells.

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Disclosure Statement

None.

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