Urol Res (2012) 40:131–141
DOI 10.1007/s00240-011-0420-8

ORIGINAL PAPER

The construction of an oxalate-degrading intestinal stem cell population in mice: a potential new treatment option for patients with calcium oxalate calculus

Zhiqiang Chen · Guanlin Liu · Zhangqun Ye · Debo Kong · Lingfang Yao · Hui Guo · Weimin Yang · Xiao Yu

Received: 14 October 2009 / Accepted: 9 August 2011 / Published online: 3 September 2011

Abstract About 80% of all urological stones are calcium oxalate, mainly caused by idiopathic hyperoxaluria (IH). The increased absorption of oxalate from the intestine is the major factor underlying IH. The continuous self-renewal of the intestinal epithelium is due to the vigorous proliferation and differentiation of intestinal stem cells. If the intestinal stem cell population can acquire the ability to metabolize calcium oxalate by means of oxc and frc transgenes, this will prove a promising new therapy option for IH. In our research, the oxalate-degrading genes of *Oxalobacter formigenes* (Oxf)—the frc gene and oxc gene—were cloned and transfected into a cultured mouse-derived intestinal SC population to give the latter an oxalate-degrading function. Oxf was isolated and cultivated and the oxalate-degrading genes—frc and oxc—were cloned. The dicistronic eukaryotic expression vector pIRES-oxc-frc was constructed and transferred into the mouse stem cell population. After selection with G418, the expression of the genes was identified. The oxalate-degrading function of transfected cells was determined by transfection into the intestinal stem cell population of the mouse. The change in oxalate concentration was determined with an ion chromatograph. The recombinant plasmid containing oxc and frc genes was transfected into the stem cell population of the mouse and the expression of the genes found normal. The cell population had acquired an oxalate-degrading function. The oxc and frc genes could be transfected into the intestinal stem cell population of the mouse and the cells acquired an oxalate-degrading function.

Keywords *Oxalobacter formigenes* · Idiopathic hyperoxaluria · Frc gene · Oxc gene · Intestinal stem cell

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Introduction

Urological calculus is a worldwide disease, of which calcium calculus is the most common type. 4–5% of all urological patients suffer from this disease, the majority of whom are young or middle-aged. About 60–80% of the stones are calcium oxalate calculus, which is mainly caused by idiopathic hyperoxaluria (IH). The stone-forming, contributing factors of calcium oxalate calculus are: hyperoxaluria, hypercalciuria, hyporcitrauria, hyperuricuria and the lack of an inhibitor, the foremost contributing factor being hyperoxaluria [1], and the increased absorption of oxalate from the intestine is the major factor behind IH [2].

Oxalic acid is an end product that cannot be further metabolized and is highly toxic. It has been suggested, but not proven, that oxalic acid is an important component in other diseases, such as irritable bowel syndrome and vestibulitis [3–6]. Currently, considerable attention has been focusing on the presence or absence of oxalate-degrading microorganisms, in particular, the gut-associated bacterium *Oxalobacter formigenes* (Oxf) in the intestines of vertebrate animals and human beings. Recent evidence suggests that Oxf have an important symbiotic relationship with their vertebrate hosts by regulating oxalic acid absorption in the intestine as well as oxalic acid levels in the plasma [2, 7, 8].

Oxf are microorganisms that dwell in the gut of animals or humans and have a powerful oxalate-degrading capability [9]. Oxf's oxalate-degrading genes are oxc and frc, which, respectively, express as oxaly1-coenzyme A decarboxylase (OCoAD) and formy1-coenzyme A transferase (FCoAT) and were first purified by Baetz and Allison in 1989 [10] and 1990 [11], respectively. Recent evidence has suggested that Oxf may offer a new treatment option for patients with primary and secondary hyperoxaluria [12]. It has been reported that Oxf can be transplanted successfully into the intestines of patients with primary hyperoxaluria by oral application, but the long-term post-treatment follow-up of 1–2 years showed that constant intestinal colonization is not achieved by this method in most patients [12], thus potentially limiting its use.

The intestinal epithelium is the main area for nutrition absorption and it continually self-renews during the animal's life because of the intestinal stem cells that continue vigorously proliferating and differentiating. The stem cells are at the bottom of intestinal crypts. The necrosis and apoptosis of the epithelium cells keep a balance between this proliferation and the differentiation [13, 14]. If the stem cell population can acquire the capacity to metabolize oxalate through the oxc and frc transgenes, this will present a promising new therapy option for hyperoxaluria, especially for IH. But oxc and frc are prokaryotic genes generally expressed in bacteria, and it is uncertain whether the oxc and frc genes can be expressed in eukaryocytes and still continue their oxalate-degrading enzyme activity. Recently, Ye et al. [15] confirmed that these two genes could also generally express in eukaryocytes—human embryo kidney 293 (HEK 293) cells.

In this study, we constructed a dicistronic eukaryotic expression vector containing the coding sequence of the oxc and frc genes and transfected the mouse intestinal stem cell population (isolated from a BALB/c mouse and identified by the immunohistochemistry method) with this vector. Moreover, the expression of OCoAD and FCoAT in mouse the intestinal stem cell populations was determined by a series of assays. The oxalate-degrading function of the cells was determined by ion chromatography in the hope that it could be used to treat hyperoxaluria, especially IH-caused diseases.

Materials and methods

Bacteria

The Oxf were isolated from human fecal specimens and cultivated under anaerobic conditions at 37°C in OxB (a liquid culture medium with sodium oxalate) and OxD (an agar culture medium with Ca-oxalate crystals). The anaerobic culture methods described by Allison [16] and Dawson [17] were employed. 100–200 mg fresh feces sample was collected from a healthy Chinese. The feces was then put into a tube with an OxB liquid culture medium for dilution and shaken for 3–5 min. Each sample preparation was gassed with CO_2 to maintain the anaerobic condition. A 0.1 ml sample was then taken from the tube and introduced into the OxD agar culture medium, which had been made anaerobic. CO_2 was immediately introduced into the tube, which was placed in ice water and rapidly shaken. The OxD agar culture medium immediately solidified and evenly coated the tube wall. At the same time, a CO_2 column formed within the tube. It was then cultivated under anaerobic conditions at 37°C for 7 days or more. The colonies in the OxD agar culture medium that emerged in approximately 7 days were picked and streaked on the plate of the OxD agar culture medium. After 5–14 days' incubation, subsequent colonies were transferred onto the OxB liquid culture medium for the cultivation of Oxf. *Escherichia coli* and enterococci standard strains were inoculated in the OxB liquid culture medium as negative controls and in the OxB liquid culture medium as blank controls. All four of the groups were cultivated under anaerobic conditions at 37°C for 10 days [16, 17].

The morphologic and functional identification of *Oxalobacter formigene* strains was performed by stereoscopic microscope, a 752 ultraviolet spectrophotometer and ion chromatography (Dionex, DX-80, USA). The strain was kept at the urologic laboratory of Tongji Hospital at −80°C.

PCR for oxc and frc genes

The bacterial genomic DNA, as a template for following PCR amplification, was extracted from Oxf cells using a Takara bacterial genomic DNA extraction kit (Takara, Dalian, People's Republic of China). Oligo 6.0 and Primer Premier 5.0 software were used for designing primers. oxc (GenBank: M77128) primers were: upstream primer oxc F

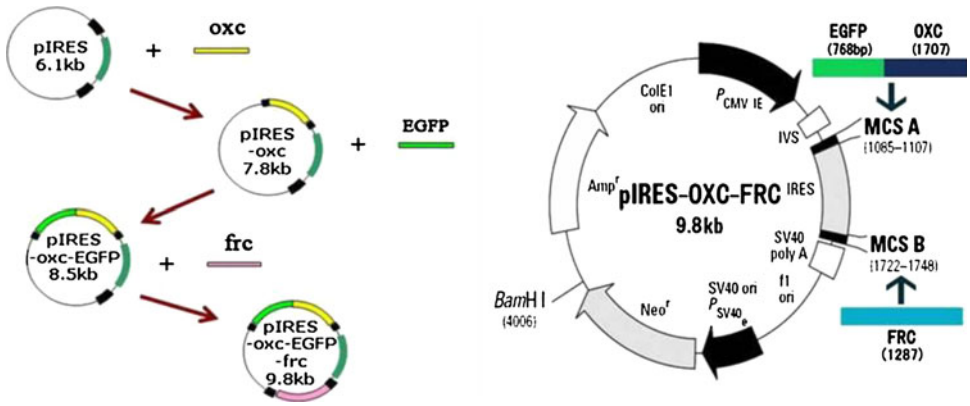

Fig. 1 Construction of the eukaryotic expression vector

for the 5′-GAATTCATGAGTAACGACGACAATGT-3′; oxc R primer for the 5′-GAATTCTTATTTCTTGCCA ACTTTAC-3′; oxc seq F primer sequences for the 5′-ACT TGCCAGCAAAACTGTTC-3′. frc (GenBank: U82167) primers were: upstream primer frc F for the 5′-GAATTC ATGACTAAACCATTAGATGG-3′; downstream primer frc R is 5′-GAATTCTCAAACTACCTGTTTTGCAT-3′; sequencing primer frc seq F for the 5′-TCACAAAA CCGGCCGTGGTC-3′. The base pair length of oxc and frc are 1707 and 1287. *Eco*RI and *Bam*HI restriction sites were incorporated into the 5′ end of the forward and reverse primers, respectively. PCR was carried out for one cycle at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C and another 10 min at 72°C for extension. The products were electrophoresed in 1% agarose gel for documentation. Sequence analysis also demonstrated that the products were the genes of interest. The two genes were inserted into the plasmid pMD18-T (Takara, Dalian, People's Republic of China), and finally the inserted segments were identified as oxc and frc by sequencing. The recombinant plasmids were named pMD18-T-oxc and pMD18-T-frc.

Construction of dicistronic eukaryotic expression vector

The dicistronic eukaryotic expression vector pIRES (Clontech, USA) was employed to subclone the genes of interest. The plasmids and the pMD18-T-oxc were digested with *Eco*RI and *Bam*HI restriction enzymes and then size separated by electrophoresis. Separated DNA fragments of pIRES (6.1 kb) and oxc (1.7 kb) were extracted from gel slices and then ligated overnight. Recombinant plasmids were identified by *Eco*RI and *Bam*HI restriction enzyme digestion and DNA sequencing. The recombinant plasmid was named pIRES-oxc. An EGFP (a mutation of wild-type GFP optimized for brighter fluorescence and higher expression in mammalian cells) gene segment was cut from pEGFP-C1 (Clontech, USA) by Nhe I/Xho I restriction enzymes and size separated by electrophoresis. The pIRES-oxc were also digested with Nhe I/Xho I restriction enzymes and then size separated by electrophoresis. Separated DNA fragments of the recombinant plasmid (7.8 kb) and EGFP (0.7 kb) were extracted from gel slices then ligated overnight. Theses plasmids were identified by Nhe I/Xho I restriction enzyme digestion and DNA sequencing and named pIRES-oxc-EGFP. The pIRES-oxc-EGFP was digested with Sal I and the pMD18-T-frc was digested with *Eco*RI and *Bam*HI. Separated DNA fragments of pIRES-oxc-EGFP (8.5 kb) and frc (1.3 kb) were extracted from gel slices. A dicistronic eukaryotic expression vector, which was named pIRES-oxc-frc, was constructed by adhesive terminal ligation after the terminal ends were filled up. As is shown in Fig. 1, the EGFP gene segregated segment formed pEGFP-C1 with the help of the restricted enzyme and connected to site MCS A of pIRES, and the oxc and frc gene segments were inserted into sites MCS A and MCS B of pIRES, respectively. An empty vector which named pIRES-EGFP was constructed with the EGFP inserted into MCS A of pIRES by similar method. The empty vector was used for control.

Construction and identification of normal mouse intestinal stem cell population

The intestine of the embryo was obtained from a 17-day pregnant BALB/c mouse (SPF, experimental animal center of Tongji Medical College, Huazhong University of Science and Technology, People's Republic of China). SPF means "Specific Pathogen Free" and it refers to animals without specific microorganisms or parasites in the body, but non-specific microorganisms and parasites are permitted. The mice was killed by cervical vertebra dislocation followed the institutional guide lines. The intestinal tissue was excised after the mesentery was split and digested by alidase and XI collagenase (Sigma, USA). The digestion was terminated by centrifugation 20 min later. The cells were then precipitated several times to remove the fibroblasts and the smooth muscle cells. After the centrifugation, the cells were re-immersed in the culture medium, which we

named stem cell culture medium, that contained DMEM, newborn bovine serum, insulin, Glutamyl transpeptidase and epidermal growth factor [14]. The cells were then implanted in the plastic culture bottle. The culture medium was exchanged 48 h later, and then exchanged every 72 h. The cells were identified by the immunohistochemistry method with anti-cytokeratin peptide 18 (Sigma, USA) and anti-vimentin (Sigma, USA) at the No. 4 and 7 passage.

Colony formation assay

The experiment included two groups: an intestinal stem cell population group and an IRD98 cell line (Shanghai Institude of cytobiology, Academia Sinica, People's Republic of China) group. Trypan blue staining was used for counting living cells. When the intestinal stem cell population was in the first passage, the cells were diluted by our stem cell medium. Cells were seeded in 24-well plates with 400 cells per well. Stem cell culture medium was added and the culture medium was changed every 3 days. 5 days later, the number of clones containing more than 32 cells was counted. At the 8th day, the cloned cells of the above 2 groups were dispersed by trypsin and seeded in 24-well plates with 400 cells per well again. Stem cell culture medium was added and the culture medium was changed every 3 days. 5 days later, the number of clones containing more than 32 cells was counted for calculating the clone formation rate of the third passage cells. The above method was repeated to detect the clone formation rate for second–seventh passage cells. The test was performed to compare the clone-forming ability between the two groups.

Detection for proliferative potential

The experiment included two groups: a fourth passage intestinal stem cell population group and an IRD98 cell group. Trypan blue staining was used for counting living cells. The cells were diluted by our stem cell medium. Cells were seeded in 96-well plates with 400 cells per well. A 100 μl stem cell culture medium was added to each well and changed every 3 days. A 2 mg/ml MTT solution was added (50 μl/well) for culturing for 4 h. Then the medium in the well was removed and a DMSO solution (150 μl/well) was added. The culture plate was placed 10 min on the microplate oscillator for shaking and the crystals were dissolved. The OD value (detection wavelength of 570 nm) was detected by a microplate reader. The results were recorded and a chart drawn.

Gene transfer and cell culture

The normal mouse intestinal stem cell population was employed to express the oxc and frc genes transferred by lipofection. The cells were seeded onto 24-well plates (Corning, USA) at a density of 1×10^5 cells per well. After 24 h of incubation, transfection was performed by Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. Plasmids (0.8 μg) (pIRES-oxc-frc) and 2.0 μl liposome were involved in the lipofection for one well. After 48 h of culture, the genetically altered mouse intestinal stem cell population was cultured with 800 μg/ml G418 (an aminoglycoside antibiotic and the most commonly used resistance screening reagent for stable transfection in molecular genetic test [15], GIBCO, USA) for 2 weeks and then 400 μg/ml for 3 weeks. Green fluorescence was observed under a fluorescence inverted microscope, and the percentage of EGFP-positive cells detected at 48 h reflected the transfection efficiency of the cells. Using the similar method, a group of cells expresses the empty vector (pIRES–EGFP) were constructed as a control group.

RT-PCR analysis

Total RNA of transgenic cells (2 μg) was applied to synthesize cDNA with a one-step RT-PCR kit (Promega, USA) according to the manufacturer's instructions, and then 1 μl cDNA was used as the template for subsequent PCR amplification. After 5 min at 95°C, 30 cycles of PCR with parameters of 95°C for 30 s, 50°C for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min, was performed to amplify cDNA of the oxc and frc genes. Two pairs of oligonucleotide primers were synthesized as follows: the oxc gene, forward primer (5′-GGCCAGAC CATTTCCGTAGAA-3′), reverse primer (5′-TGCTGC ATCAGCCAGTTCAGA-3′); the frc gene, forward primer (5′-GCACTGGACCGTATGGGCTTT-3′), and reverse primer (5′-CGGTTTTGTGACGCATTTCCA-3′). The PCR products were 0.25, and 0.17 kbp, respectively.

Western blot analysis

The total proteins of transgenic cells (50 μg) harvested from each sample were subjected to a 10% SDS–PAGE gel electrophoresis for 2 h and then electro-transferred to a nitrocellulose membrane. Blocked with 5% skimmed milk for 1 h, the membrane was probed with the monoclonal antibodies against OCoAD and FCoAT (1:100, Qulax, USA), and a primary rabbit polyclonal antibody against human β-actin (1:500, Santa Cruz, USA) and diluted in TBST overnight at 4°C. After being washed three times for 10 min each, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37°C. After being washed again, it was reacted with enhanced chemiluminescence reagent (Pierce, USA), wrapped with film and exposed.

Oxalate-degrading function of the transfected normal mouse intestinal stem cell population

The oxalate-degrading function of the transfected normal mouse intestinal stem cell population was estimated by determining the change of oxalate concentration in the medium. Sodium oxalate (Sigma-Aldrich, USA) was dissolved in PBS and filtered (to sterilize). The normal mouse intestinal stem cell population, the transgenic cells (pIRES-oxc-frc transfected) and the cells express empty vector (pIRES-EGFP transfected) were seeded onto 8 wells in 24-well plates at a density of 5×10^4 cells per well respectively. The DMEM (GIBCO, USA) medium was also injected into the other eight wells as the blank group. The solution was prepared as follows: sodium oxalate stock solution preparation methods: 2.7 g sodium oxalate was dissolved in a 100 ml phosphate buffer solution (PBS) and, after filtration, the solution was subdivided for usage.

An appropriate volume of filtered sodium oxalate stock solution was added to the DMEM cell culture medium containing a calculated 10% calf serum with a final concentration of sodium oxalate of 2.7 g/L. After 48 h of culture at 37°C, the medium in 24 wells was replaced with DMEM supplemented with 2.7 g/L sodium oxalate. The latter was collected at different time points (1, 2, 3, 4, 5 days) and filtered by a 0.2-μm membrane. Then the filtrate was injected into a DX-80 ion chromatograph (Dionex, USA) to determine the concentration of sodium oxalate. The ion chromatographic column included: CG12A (3×30 mm) guard column and CS12A (3×150 mm) analytical column, CSRS-II electron suppressor, and conductivity detector. A wash solution was prepared from 8 mM Na_2C0_3, l mM $NaHCO_3$ and 2,000 ml of deionized water (18.2 MΩ). Regenerated liquid was prepared from a 4.1 ml concentrated sulfuric acid solution and 2,000 ml deionized water. Before using, DL-01 solvent filters were used. The ambient temperature was 10–28°C. The carrier gas was nitrogen with a pressure of 0.2 Mpa. The maximum pump pressure was 21 MPa (3,000 psi) and the actual running pressure was about 1,700 psi. The background conductance was about 28 μs. The flow rate was 0.5 ml/min and the injection volume was 10 μl. Peaknet6.0 software was used for data processing. A standard curve was drawn automatically and the concentration of oxalic acid was calculated.

The IC system consisted of a Waters 501 pump, a Rheodyne 7125 injector, a Waters 431 conductivity detector, an Alltech ERIS conductivity suppressor, and an Alltech All-Sep (10 cm) anion exchange column. The mobile phase used consisted of 0.85 mmol/L $NaHCO_3$, 0.9 mmol/L Na_2CO_3 at a flow rate of 1.2 mL/min [18].

Statistical analysis

The statistical software package SPSS 12.0 was used to analyze the data. The χ^2 test was performed to compare frequencies among the groups. P values <0.05 were considered statistically significant.

Results

Isolation and cultivation of Oxf and PCR for oxc and frc genes

The colonies of Oxf were observed during the 7-day incubation at 37°C with a CO_2 atmosphere in an OxB medium. The typical colonies were either white dots or fusiform and differed from each other in size (0.1–0.5 mm × 1.0–3.0 mm). Each colony was surrounded by a clear zone with a maximum diameter of 4.5 mm. It could be seen that the bacteria were curved, arc/rod-shaped, Gram-negative and measured 1.0–1.6 μm × 3.0–6.5 μm but without any motility, and spores were formed. It showed that the concentration of oxalate in the OxB medium gradually decreased with culture time and the increase in Oxf ($P < 0.05$, Fig. 2). Comparing the sequence of nucleic acid of the frc gene with that reported by the gene bank, the matching rate was 95.88% (1,234/1,287), while the matching rate of protein was 99.07% (424/428). The oxc gene of the Chinese intestinal Oxf was cloned. Comparing the sequence of nucleic acid of the oxc gene with that reported by the gene bank (the oxc gene, ID: M77128, from a typical strain and the frc gene, ID: U82167, from a typical strain, were from GenBank) the matching rate was 93.61% (1,598/1,707), while the matching rate for protein was 97.18% (552/568).

Construction and identification of normal mouse intestinal stem cell population

The cells began to adhere after 48 h. At this stage, 30% of the clones were found to be lined with a homogeneous population of large, undifferentiated epithelial-like cells, while 20% of the clones were lined with a layer of flattened epithelial-like cells. The remaining clones (50%) appeared to be lined with both types of epithelial-like cells. Little mechanocyte-like cells were also found. Cells of the No. 3 and 4 passages were the most active. Subsequently, the number of stem cells began to decrease, in the end the cells apoptosized (No. 7 and 8 passage, 6–7 weeks). At No. 4 and No. 7 passage, the cells were identified by Anti-Cytokeratin Peptide 18, and the results were positive (Fig. 3). The results of the anti-vimentin identification at No. 4 and No. 7 passage were negative.

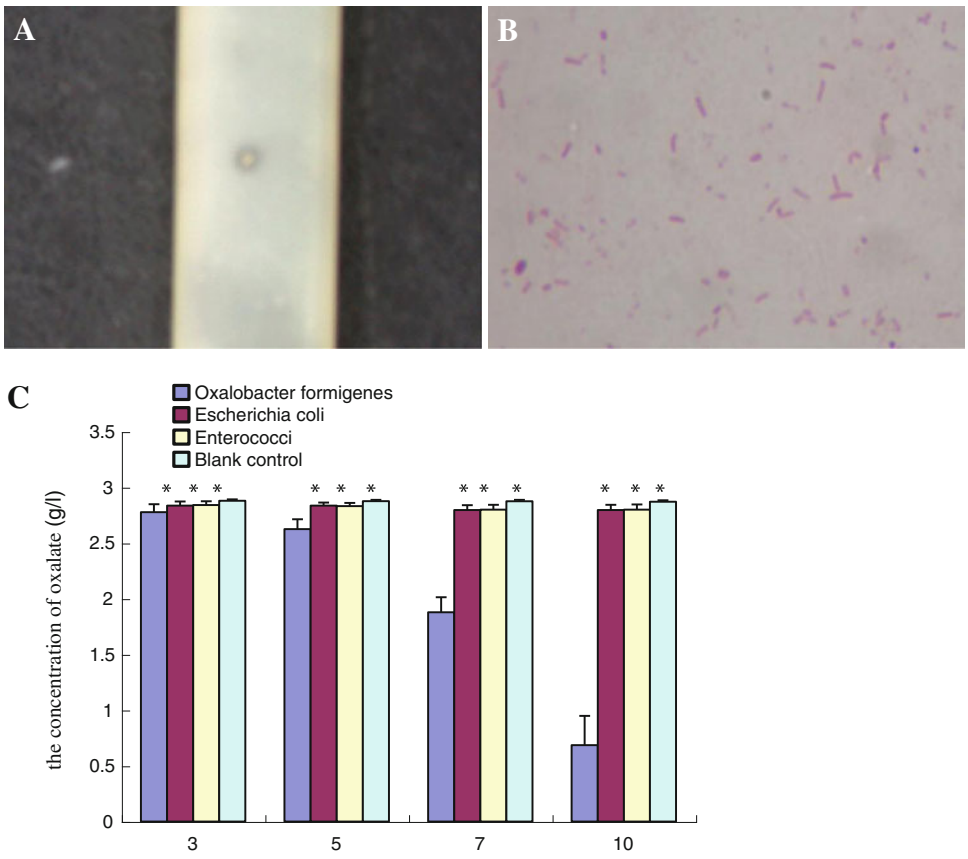

Fig. 2 Isolation and cultivation of Oxf. **a** The morphological characteristics of Oxf in the colony. **b** The morphological characteristics of *Oxalobacter formigenes* bacteria (×1,000). **c** The concentration of oxalate of four groups at 3, 5, 7 and 10 days after anaerobic cultivation

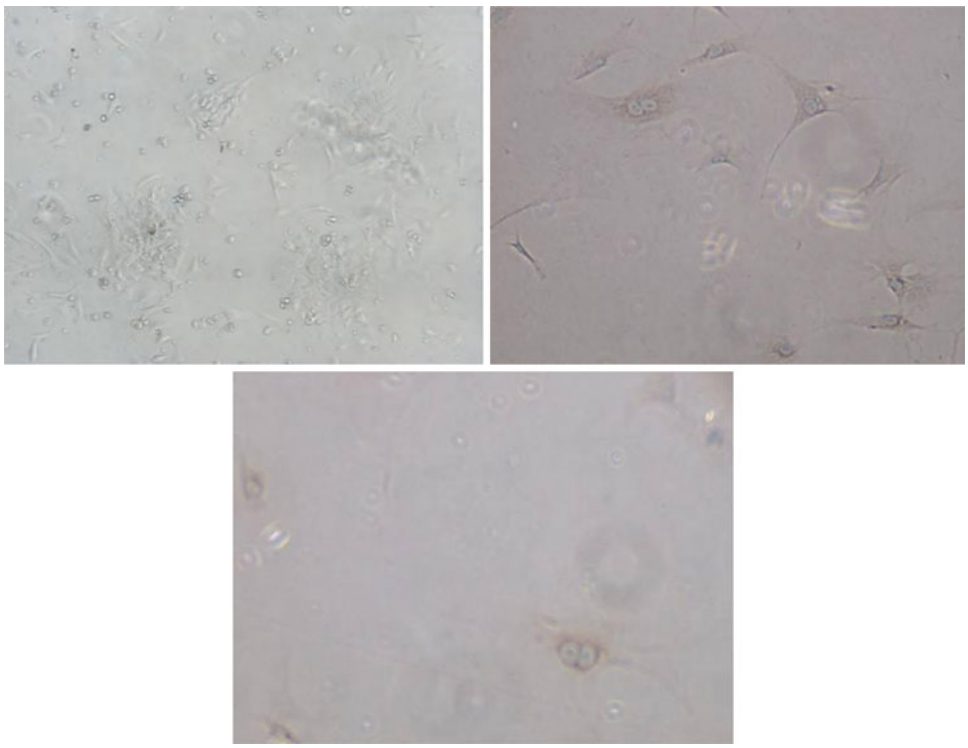

Fig. 3 Growth morphology of the mouse intestinal stem cell population cultures

Table 1 Clone formation rate of the two kind of cells

	Clone formation rate (%)					
	2nd passage	3rd passage	4th passage	5th passage	6th passage	7th passage
IRD98	60.71 ± 1.16	60.94 ± 0.73	60.75 ± 0.94	60.78 ± 0.84	60.72 ± 0.95	60.63 ± 1.20
Intestinal stem cell population	61.34 ± 0.73	72.34 ± 1.93	74.06 ± 1.76	65.40 ± 1.05	39.19 ± 0.44	26.84 ± 1.03

Colony formation assay results showed that the colony formation rate of second-passage intestinal stem cell populations isolated in our study was almost the same as IRD98 cells, the colony formation rate of the third and fourth passage cells was significantly higher than IRD98 cells. After the fifth passage, although the colony formation rate was still higher than IRD98 cells, but the colony formation rate declined because the cells began to apoptosize (Table 1, $P < 0.05$). Since the sixth passage the colony formation rate of our cells was less than IRD98 cells, at the eighth the cells we isolated and cultured were almost died. This suggested that the intestinal stem cell population which was isolated and cultured in our study had a very strong self-renewal capacity.

MTT results showed that 2 days after the fourth passage(most active passage) cells were seeded in the stem cell culture medium, the OD value, which could reflect the cell number, of intestinal stem cell population was significantly higher than that of IRD98 cells (Fig. 4, $P < 0.05$). The cell proliferation curve showed that the intestinal stem cell population proliferated slowly from the seventh day onward, while IRD98 cells proliferated also slowly from day 7 onward, because the wells were almost full of the cells. This suggested that the intestinal stem cell population isolated and cultured in our study had a high proliferative potential.

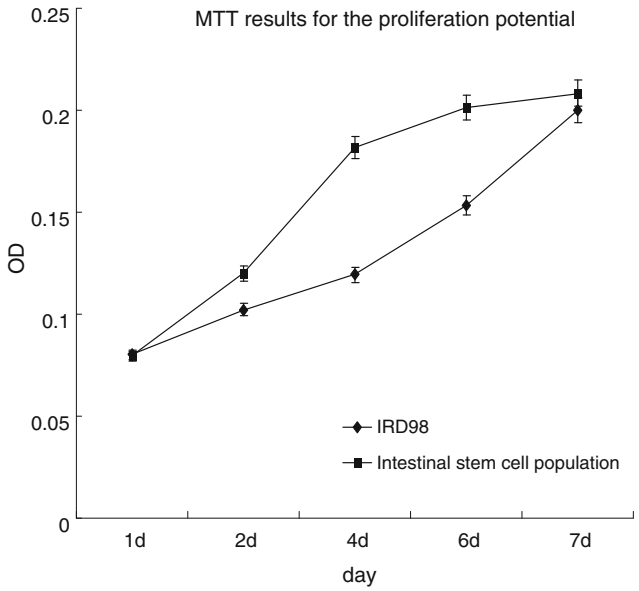

Fig. 4 Detection for proliferative potential

Stable transfection with pIRES-oxc-frc in the normal mouse intestinal stem cell population

pIRES-oxc-frc was transferred into the normal mouse intestinal stem cell population by lipofection followed by a selective culture. 6 h after transfection, a slight green fluorescence was visible in these cells using a 488-nm blue laser excitation light under an inverted fluorescence microscope. The cells were grown for 2 days in a nonselective medium and then trypsinized followed by seeding into 6-well plates. The cells were cultured with 800 μg/ml G418 for 2 weeks. At 24 and 48 h after transfection, bright green fluorescence was observed from a few cells scattered throughout the wells, and the transfection efficiency at 48 h of pIRES-oxc-frc was 25.8%. After 3 days in a G418-selective culture, several fluorescent cell colonies formed and a few non-fluorescent cells were scattered throughout the colonies. To achieve a pure and stably transfected cell line, the single colonies of cells were picked out and seeded again at a diluted density every 3 days for 2 weeks. Subsequently, selected cells were proliferated with 400 μg/ml G418 for 3 weeks. At this time, almost all cells exhibited bright green fluorescence as shown in Fig. 5.

RT-PCR analysis of the transfected oxc and frc genes expression in the mouse intestinal stem cell population

RT-PCR was performed to confirm the expression of oxc and frc genes. Oxc or frc mRNA expression in the mouse intestinal stem cell population transfected with pIRES-oxc-

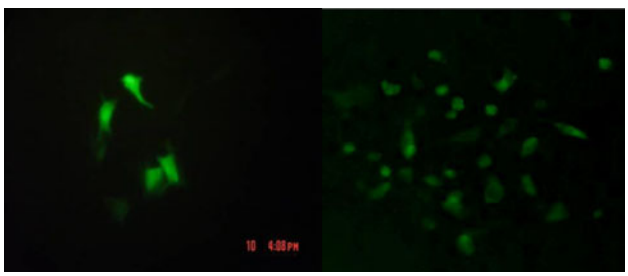

Fig. 5 The transfected cells identified by green fluorescence. At 48 h after transfection, the transfection efficiency of pIRES-oxc-frc was 25.8% (*left*). At 5 weeks after transfection, almost all cells exhibited bright green fluorescence, and fusion proteins were located in the cytoplasm (*right*)

frc was demonstrated by RT-PCR. The sizes of the amplified fragments were 0.25 kb (oxc) and 0.17 kb (frc). The result showed the transfected mouse intestinal stem cell population could stably express oxc and frc genes at the mRNA level (Fig. 6).

Western blot analysis of OCoAD and FCoAT protein expression of the transgenic mouse intestinal stem cell population

Protein immunoblot analysis was performed with an anti-OCoAD monoclonal antibody and an anti-FCoAT monoclonal antibody. The result showed the pIRES-oxc-frc transfected mouse intestinal stem cell population could express OCoAD and FCoAT protein (Fig. 7).

Analysis of the transgenic mouse intestinal stem cell population's oxalate-degrading function

To confirm that the OCoAD and FCoAT expressed in the mouse intestinal stem cell population retained their enzyme activity, the concentration of oxalate in the medium was detected for 5 days. As shown in Fig. 5, the concentration of oxalate in the medium of the mouse intestinal stem cell population transfected with pIRES-oxc-frc exhibited a significant decrease, but there was no significant shift observed in the medium of other groups ($P < 0.05$, Fig. 8).

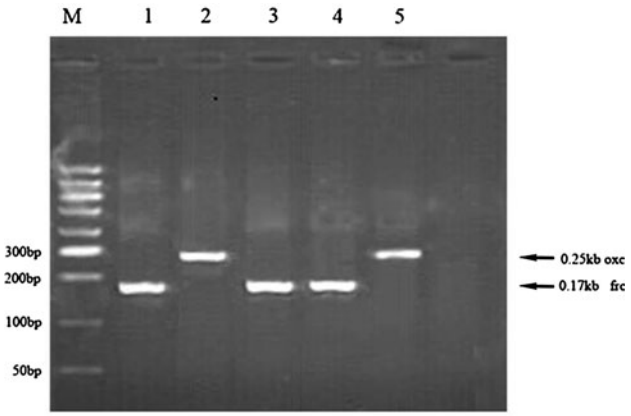

Fig. 6 The results of RT-PCR analysis showed that gene oxc (*2*, *5*, 0.25 kb) and frc (*1*, *3*, *4* 0.17 kb) were detected at the mRNA level in the stably transfected cells

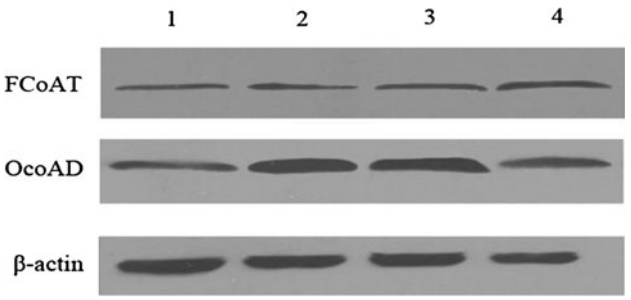

Fig. 7 The results of protein immunoblot analysis showed that OCoAD and FCoAT was detected in the stably transfected cells

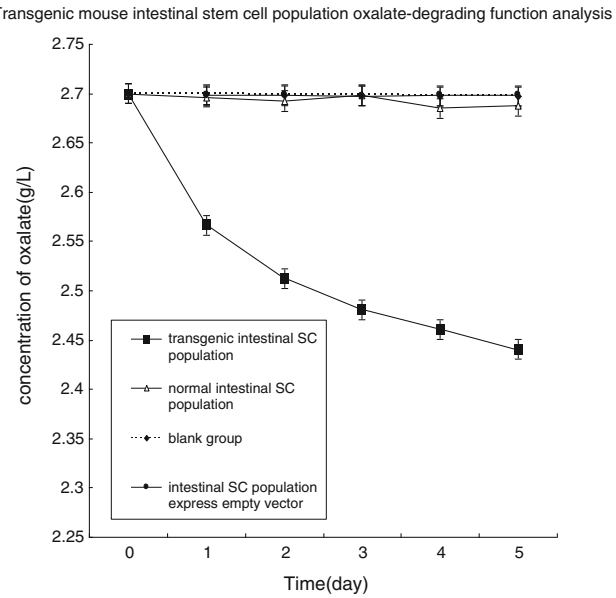

Fig. 8 Transgenic mouse intestinal stem cell population oxalate-degrading function analysis

Discussion

Oxalate is a highly toxic natural by-product of normal cellular metabolism and is ingested with many common foods and beverages, such as tea, coffee, chocolate, fruits, and vegetables [6]. There are several distinct mechanisms for oxalate catabolism present in nature, but unfortunately, no oxalate-degrading pathway has been found in the human body. If oxalate in urine increases slightly, it is diagnosed as idiopathic hyperoxaluria, which is the major mechanism of idiopathic calcium oxalate calculus. Idiopathic hyperoxaluria is one of the most important etiological factors of calcium oxalate caculus, and research in the past few years has indicated that the exogenous absorption of oxalate is the most important etiological factor in idiopathic hyperoxaluria [2].

Oxf, a group of Gram-negative, obligate anaerobic, nonmotile, rod-shaped bacteria utilizing oxalate as its exclusive source of energy, were characterized and first named by Allison et al. [16] in 1985. This anaerobic bacterium that dwells especially in the colon was able to obtain energy by oxalate-degradation [19, 20]. The key enzymes in oxalate-degradation are formyl-coenzyme A transferase (FCoAT) and oxalyl-coenzyme A decarboxylase (OCoAD),

which were encoded by frc and oxc genes [10, 11]. The intake of the oxalate relies on a membrane transport molecule that established the proper proton imbalance [10]. The decarboxylated degradation of oxalate in Oxf is a two-step enzymatic reaction catalyzed by OCoAD and FCoAT. In this anaerobic metabolic procedure, one molecule of oxalate is decarboxylated to form one molecule of CO_2 and one molecule of formate, which is coupled with ATP synthesis. FRC is encoded by the frc gene and catalyzes a transfer of CoA from formate to oxalate, which is necessary for the activation of oxalate before its decarboxylation, while OCoAD is encoded by the oxc gene and mediates an oxalyl moiety for thiamine-dependent decarboxylation to form formyl-CoA and liberate CO_2 [15]. Oxc and frc genes were transfected into *E. coli*, and the latter were made to express OCoAD and FCoAT by Lung and Sidhu in 1994 and 1997, and the products exhibited enzyme activity similar to the native enzymes [21, 22].

Since Oxf may digest enough oxalate to reduce intestinal absorption and the corresponding urinary excretion of oxalate [23], it is suggested that Oxf may offer a new treatment option for patients with primary and idiopathic hyperoxaluria [12, 24].

In our research, although the morphology of Oxf was minimally different from previous studies [16, 17], our results provided significant evidence that Oxf performed the active oxalate catabolism function in vitro. Furthermore, we also identified Oxf at the gene level.

It was reported that Oxf could be transplanted successfully into the intestin in- patients with primary hyperoxaluria using an oral application, but the long-term post-treatment follow-up of 1–2 years showed that constant intestinal colonization is not achieved in most patients [12]. So Oxf cannot be used directly as a new treatment tool.

The small intestinal mucosa is the main place where nutritive material is absorbed. The self-renewal of the intestinal epithelium mucosae continues during an organism's entire life-cycle as a result of the intestinal stem cells at the bottom of the intestinal crypts that retain the capacity for vigorous generation and differentiation. The intestinal stem cells are adult stem cells with a theoretically inexhaustible capacity for generation and differentiation [13, 14]. As the exact mechanism of oxalate intake in intestinal mucosa cells is still controversial until now, it is certain that the small intestinal mucosa is also the main place where oxalate is absorbed [15, 16]. If a stem cell population can acquire the capacity to metabolize oxalate with the transgenes of oxc and frc, this presents a promising new therapy option for hyperoxaluria, especially for IH, and calcium oxalate caculus. Oxc and frc are prokaryotic genes generally expressed in bacteria, and it is still uncertain whether the oxc and frc genes can be expressed in eukaryocytes and still perform their oxalate-degrading enzyme activity. Recently, Ye et al. confirmed that these two genes could also generally express in eukaryocytes–human embryo kidney 293 (HEK 293) cells [15]. But unfortunately, no generally accepted specific marker has yet been found in intestinal stem cells, and only cytokeratin peptide 18 was reported to express in clones with stem cells [13, 14]. Therefore, we had to identify the cells we isolated and cultured by a combined method.

In this research, the cells begin to adhere after 48 h. At this stage about 30% of the clones were found to be lined with a homogeneous population of large, undifferentiated epithelial-like cells, and about 20% of the clones were lined with a layer of flattened epithelial-like cells, The remaining clones (about 50%), appeared to be lined with homogeneous epithelial-like cells both large and flattened (Fig. 3). The shape of the cells was similar to that reported in the literature [14], and little mechanocyte-like cells were found. Cells of the third and fourth passage were the most active. Subsequently, the number of stem cells decreased and finally apoptosized. The cell population was able to survive about 7–8 passages and showed a high capacity for passage and differentiation. At the No. 4 passage, the cells were indentified by anti-cytokeratin peptide 18, the result was positive (Fig. 3). At the No. 7 passage, although the quantity declined, there were still some cells displaying positive results (Fig. 3). As reported in the literature, the cytokeratin peptide 18 was expressed in the stem cells [14], so the cells we isolated and cultured were identified as a mouse intestinal stem cell population. Since the results of the anti-vimentin identification at No. 4 and 7 passages were negative, it meant that few mesenchymal cells were found in our cell population. The immunohistochemical results agreed well with the cell shapes. Colony formation ability and proliferative potential are two important indexes to detect stem cell character. Since primary isolating and culturing adult intestinal epithelial cells from BALB/c mice is very difficult, fetal rat intestinal cell line IRD98 was used to be the control group. IRD98 is a kind of immortalized fetal rat intestinal cell line which contains a lot of stem cells and progenitor cells theoretically [13, 25]. However, adult intestinal epithelial cells also existed in IRD98. Colony formation assay and MTT results showed that compared with IRD98, the intestinal stem cell population we isolated and cultured had a stronger self-renewal capacity and proliferation potential than normal fetal rat intestinal epithelial cell line IRD98 during the most productive period of their growth ($P < 0.05$) [26, 27] (Table 1; Fig. 4). That indicated the cell population we isolated and cultured contained more stem cells, which made our mouse intestinal stem cell population had stem cell character.

Oxc and frc are prokaryotic genes and cannot usually be expressed in eukaryotic cells due to their hereditary

differentiation in the expressing control region. In our study, the dicistronic eukaryotic expression vector pIRES was employed to subclone the frc and oxc genes. Because of the internal ribozyme entry sites (IRES), pIRES is a dicistronic eukaryotic expression vector that could express two exogenous genes simultaneously in eukaryocytes (Fig. 1). But unfortunately the vector pIRES still lacks a rapid and sensitive reporter gene as a biological marker, such as the enhanced green fluorescent protein (EGFP). EGFP, a mutant of wild-type GFP, has been optimized for brighter fluorescence and higher expression in mammalian cells [28]. EGFP is considered a rapid and sensitive reporter gene as a biological marker, and the proteins that fuse to EGFP gain fluorescence while continuing to retain their native function [29]. We amplified the oxc and frc genes from genomic DNA extracted from Oxf. The explored gene segments oxc and frc in the research contained only a coded region. An EGFP gene segment was cut from pEGFP-C1. The oxc gene and the EGFP gene were inserted into the MCS A site of pIRES. The frc gene segment was inserted into the MCS B site. The recombined plasmid, which could express oxc and frc simultaneously in eukaryotic cells, was named pIRES-oxc-frc.

Subsequently, the recombinant plasmids that carried the oxc and frc genes were transferred to the mouse intestinal stem cell population by Lipofectamine™ 2000. The result showed that some of the pIRES-oxc-frc transfected mouse intestinal stem cell population expressed fluorescent green protein under a fluorescence microscope 6 h later. The number of these cells increased after 24 h and reached a maximum of 25.8% after 48 h, which showed the plasmid has been successfully transferred into the mouse intestinal stem cell population. After 5 weeks of G418-selective culturing, almost all cells exhibited bright green fluorescence, which indicated stable transfection and expression of the plasmids (Fig. 5).

In most cases the resultant EGFP chimera and the tagged protein did not affect the activity of the latter [30]. The fluorescence of the mouse intestinal stem cell population transfected with pIRES-oxc-frc showed that the proteins of interest were located in the cytoplasm (Fig. 2). An analysis of RT-PCR products by agarose gel electrophoresis confirmed the expression of the recombinant oxc and frc genes in the stably transfected cells at the mRNA level (Fig. 6). Western blot further showed frc and oxc genes had stable expression in transfected intestinal stem cell populations at the protein level (Fig. 7).

Ye et al. [15] had confirmed that these two genes could also generally express in eukaryocytes—human embryo kidney 293 (HEK 293) cells. There are several examples of the expression of prokaryotic genes in eukaryotes, such as the LacZ [31] and G418-resistance genes [30]. But it was still uncertain whether the OCoAD and FCoAT could continue their enzyme activity as exogenous proteins in a mouse intestinal stem cell population.

In our study, the change of oxalate concentration in the medium was documented with ion chromatography for 5 days to ascertain whether the OCoAD and FCoAT expressed in the mouse intestinal stem cell population continued their enzyme activity. In the medium of the transgenic cells, there was a significant decrease in oxalate, which indicated that the OCoAD and FCoAT expressed in the mouse intestinal stem cell population continued their enzyme activity and the transgenic mouse intestinal stem cell population had obtained a much better oxalate-degrading ability (Fig. 8). Since the transgenic cells had been cultured more than 5 weeks, most cells were adult small intestine epithelial cells [14, 15], indicating that the oxc and frc genes were successfully transferred into the intestinal stem cells by transgenic technology and that the transgenic stem cells could generate and differentiate into intestinal mucosa cells, which took on the oxalate-degrading function.

It is really a pity that the amount of decrease is minimal (what appears to be about 30–27 mM), the exact mechanism still needs further research, and the enzymes' difference between mammalian cell systems and Oxf maybe an important factor. Although the amount of decrease is clearly minimal with a temporal leveling off, the result still had statistical significance. The construction of an oxalate-degrading intestinal stem cell population is able to lessen the absorption of exogenous oxalate, which is very significant and might very well be a new therapy for the treatment and prevention of calcium oxalate caculus. It has been reported in the literature that some researchers have begun experimenting with intestinal cell orthotopic transplantation in rodents and have had some encouraging successes in metabolic disease gene therapy [32]. Continuing such research based on a successfully constructed oxalate-degrading intestinal stem cell population certainly will be significant in the prevention and treatment of calcium oxalate calculus.

Under general conditions, prokaryocytes genes cannot be transferred into eukaryocytes and normally expressed [33]. In our research, two genes of prokaryocytes were transferred into eukaryocytes by lipidosome. Lipidosome is a bimolecular vesicle with similar structure to a cytomembrane and can exist in nature. As is well known, despite the fact that the control regions of prokaryocytes and eukaryocytes are different, homology does exist. Gene exchange by virus could exist between prokaryocytes and eukaryocytes. But further research is needed under special conditions to determine whether lipidosome can form after cell disruption and gene exchange occur between prokaryocytes and eukaryocytes by lipidosome. The result may provide some new viewpoints and theoretical support for Darwinian evolution theory.

In conclusion, the construction of an oxalate-degrading intestinal stem cell population by transgenetic technology provides a satisfactory foundation for gene therapy for idiopathic oxalate calculus, and this research may have very important clinical value. Further discussion of gene exchange between prokaryocytes and eukaryocytes may prove to be of theoretical value for basic research.

Acknowledgments This study was supported by grants from the National Science Foundation of P.R. China (nos. 30371423, 30670820, 30872564, 81001148).

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